68TH CONGRESS : : : 1ST SESSION

DECEMBER 3, 1923–JUNE 7, 1924

HOUSE DOCUMENTS

VOL. 22

WASHINGTON : : GOVERNMENT PRINTING OFFICE : : 1924

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68TH CONGRESS, 1st Session } HOUSE OF REPRESENTATIVES { DOCUMENT No. 223

TREASURY DEPARTMENT

UNITED STATES PUBLIC HEALTH SERVICE

HYGIENIC LABORATORY—BULLETIN No. 138

APRIL, 1924

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By MAURICE I. SMITH and WM. T. McCLOSKY

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By MAURICE I. SMITH and WM. T. McCLOSKY

WASHINGTON
GOVERNMENT PRINTING OFFICE
1924

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STUDIES ON THE BIO-ASSAY OF PITUITARY EXTRACTS: CONCERNING THE USE OF A DESICCATED INFUNDIBULAR POWDER AS A STANDARD IN THE PHYSIOLOGICAL EVALUATION OF PITUITARY EXTRACTS.[1]

By MAURICE I. SMITH, Pharmacologist, and WM. T. MCCLOSKY, Assistant Pharmacologist, Hygienic Laboratory, Washington, D. C.

INTRODUCTION.

It is needless to enter here into a discussion concerning the necessity of accurately standardizing extracts prepared from the infundibular lobe of the pituitary gland, to insure uniformity in potency of the various commercial products. This has been sufficiently emphasized by various workers in this field, and the matter has been considered of so great importance that the ninth revision of the United States Pharmacopœia adopted a method for the bio-assay of pituitary extracts and made it obligatory that the commercial products conform to a certain standard (*1*). Although several years have elapsed since this went into effect, pituitary extracts from various commercial houses to-day show as much variation in potency as prevailed before the method of assay was adopted. Obviously, either the commercial firms failed to adhere to the prescribed standard, or the standard or method is unreliable. It appears that there is truth in both deductions. Several years of experience with this method of assay of pituitary extracts convinced workers in this field of the unreliability of the standard (Beta-iminazolylethylamine, or briefly, histamine) originally proposed by Roth in 1914 (*2*). Expressions of dissatisfaction with the standard are found in the publications of Fenger (*3*), Pittenger and Vanderkleed (*4*), Eckler (*5*), and others. In 1918 Spaeth wrote in reference to this standard that his "experiments confirm the evidence that histamine, on account of its deterioration and for other reasons, is not a practical standard" (*6*). Consequently we find that a commercial firm standardizes its pituitary extracts by a method which it finds most suitable, without regard for what other manufacturers do, and thus one firm puts out extracts of a potency that bears no relation whatever to that of extracts made by other firms.

[1] Manuscript submitted for publication March 18, 1923.

In 1918 Spaeth (*6*), not being satisfied with histamine as a standard for the bio-assay of pituitary extracts, suggested potassium chloride as a standard. He based this upon his observation that the reaction of the isolated uterus of the guinea pig to potassium chloride is qualitatively of the same order as that to pituitary extracts. Spaeth's experiments led him to believe that there is also a very close quantitative relationship in the reaction of the isolated uterus of the guinea pig to certain concentrations of potassium chloride and of pituitary extracts. This did not find confirmation in the hands of Nelson (*7*). During the months of March and April, 1922, one of us (M. I. S.) carried out a series of observations on the use of potassium chloride as a standard, and it was found that out of a series of experiments upon the isolated uterus of the guinea pig with a given pituitary extract it was possible to get a number of experiments yielding fairly concordant results, giving a definite ratio of activity between the extract and potassium chloride. Many experiments, however, showed variations from the established ratio all the way up to 500 per cent. It has not been possible to define clearly the conditions under which consistent results could be obtained with uniformity and it was therefore concluded that there is no constant parallelism in the irritability of different uteri or of the same uterus under different conditions toward potassium chloride and pituitary extracts.

Perhaps the clearest argument against the use of both histamine and potassium chloride as standards in the bio-assay of pituitary extracts was brought out recently by Burn and Dale (*8*) in a publication which appeared at a time when the work detailed in this paper had progressed so far as to enable us to draw some very definite conclusions.

We believe that any attempt at utilizing an artificial standard for the bio-assay of pituitary extracts would be doomed to failure unless it were known definitely that the mechanism of pharmacologic action of the standard and of pituitary extracts on the uterine muscle, the test object, were identical in every respect. This is admittedly impossible in our present state of knowledge, for even if we had definite knowledge of the action of the proposed standard we should have no exact information as to the mode of action of pituitary extracts on the uterine muscle. Much less do we know what influence slight deviations in the composition of the medium bathing the isolated uterine segment might have upon its reactions to pituitary extracts. In our judgment preparations from the pituitary gland alone, and, since the chemistry of its active principles is virtually unknown, only preparations that represent the entire infundibular lobe, should be used as a standard for the physiological assay of

pituitary extracts by the isolated uterus of the guinea pig, which, as is generally recognized, is the most useful test object.

With this in view we have attempted to discover a preparation of the infundibular portion of the pituitary gland which would show a reasonable degree of uniformity in potency, which would keep a reasonable length of time without deterioration, and which could be prepared without an undue amount of chemical manipulation.

THE METHOD AND TECHNIC OF THE BIO-ASSAY OF PITUITARY EXTRACTS.

In carrying out the assay of pituitary extracts we have used the method described by Dale and Laidlaw in 1912 (*9*), with some modifications which we have found very helpful. A diagram and detailed description of the apparatus in our use follows.

The outer warming chamber (A) is of galvanized iron and measures 15 inches in length, 7 inches in width, and 6½ inches in height. A constant temperature of 38° C. is maintained by means of a microburner, connected with a Roux bimetallic gas thermoregulator (B). The inner vessel (C) containing Locke's solution and the uterine horn is made of an ordinary Liebig condenser. The lower end serves the purpose of draining off the bathing fluid after the reaction to a given dose of an extract is completed. Through the side tube (D) fresh Locke's solution is allowed to run in. This solution is warmed to the required temperature in the glass coils (E) and a Fresenius nitrogen bulb (F), which are both connected in series with the reservoir of Locke's fluid.[2] The volume of the coils and flask is approximately twice that of the vessel (C), which holds a little over 100 c. c. One end of the horn of the uterus is attached by means of a fine hook to the lower end of the glass tube (G), which is bent down at (H) and drawn out to a capillary tip. The stream of oxygen bubbles conveyed to the fluid in C is thus diverted from the muscle. The Wolff bottle (M) has a capacity of about 500 c. c. and contains a 2 per cent solution of sodium bicarbonate in distilled water. The oxygen passes through this bottle before entering vessel (C).

During the early part of this work we were frequently confronted with the difficulty of the uterine preparation rapidly increasing in sensitiveness toward pituitary extracts, so that successive reactions to a given dose of the extract soon increased to a maximum. This made it impossible to get more than a very few, and often only approximate, comparisons, and at the end of an experiment there would still be some doubt as to the relative potency of the two extracts examined. We suspected that the oxygen which we were using contained some impurity which tended to augment the tone

[2] The senior author had seen a similar warming glass coil arrangement in use in the pharmacological laboratory of the University of Michigan.

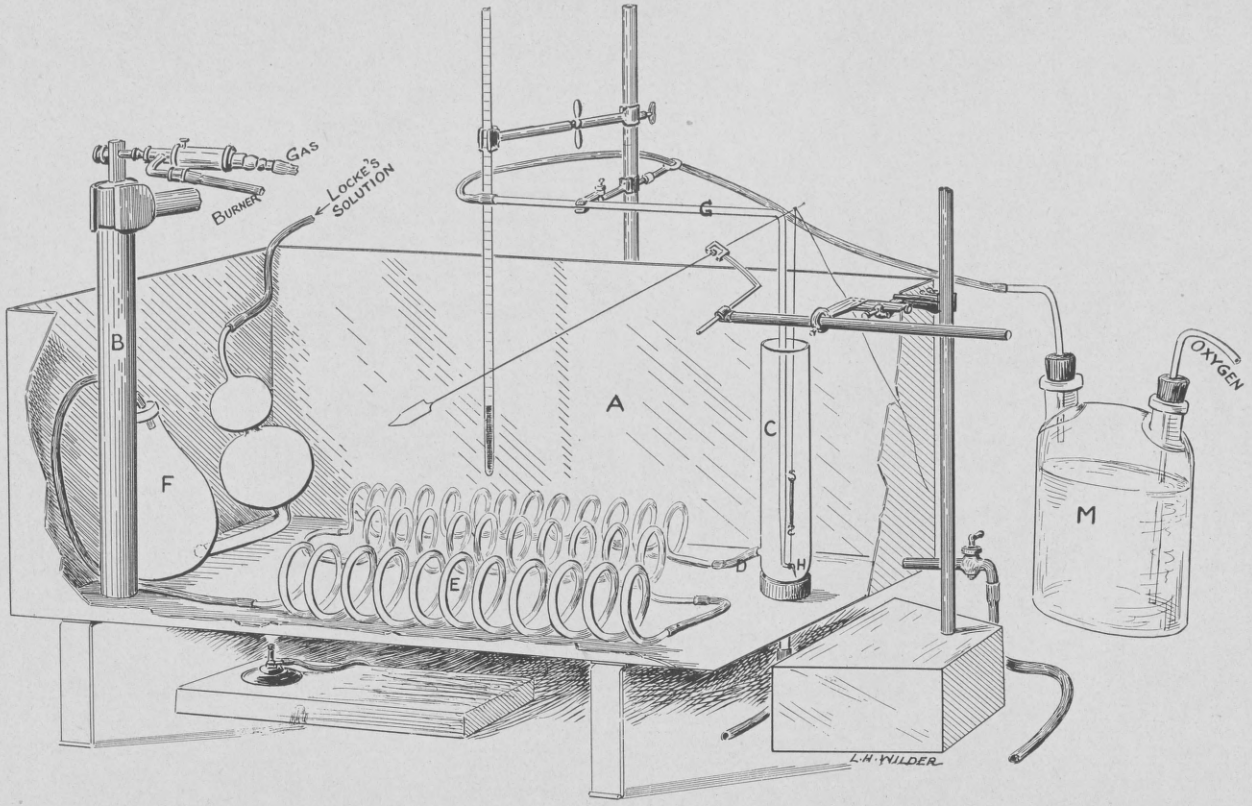


Diagram of perfusion apparatus for the bio-assay of pituitary extracts.

of the uterine muscle and to sensitize it to pituitary extracts. We then attempted to wash the oxygen in an effort to free it of its suspected impurity, and we discovered that by washing the oxygen through a solution of sodium bicarbonate we obtained incomparably better results. We then proceeded to determine by means of indicators, such as are used in hydrogen ion concentration work, whether there was a measurable amount of acidity in the oxygen. The results were negative, but it was observed that as the oxygen bubbled through the solution of sodium bicarbonate a small amount of carbon dioxide was blown over with it, and it was concluded that it is the small amount of carbon dioxide admixed with the oxygen that tends to maintain the tone and irritability of the uterine muscle at a uniform level. Whether the effect is due to the direct action of carbon dioxide on the uterine muscle or to an indirect effect owing to the maintenance of an optimum balance of ions in the bathing fluid can not at present be answered.

We did not attempt to work out in detail the optimum concentration of carbon dioxide. This would obviously require a very large number of experiments. In our experience 500 c. c. of a 2 per cent solution of sodium bicarbonate, renewed every 7 to 10 days, has given us very good results.

We have used throughout this work guinea pigs weighing not much over 250 gm., nor under 180 gm., generally 200 to 240 gm. We are convinced, however, that the age of the animal is a far better guide than its weight. We have frequently met with useless preparations obtained from small guinea pigs, weighing not much over 200 gm., especially if brought into the laboratory from outside breeders. We therefore use as a routine, guinea pigs of our own stock, which are weaned and segregated at the age of 10 to 14 days. At the age of 3 to 5 weeks such guinea pigs yield very useful preparations. It is seldom if ever that uteri obtained from such animals have to be discarded. Trendelenburg and Borgmann (*10*) have called attention to the great convenience of working with quiescent uteri obtained from young virgin guinea pigs and we can confirm these authors. Such uteri require very little weighting, are easily adjusted, and show a considerable degree of sensitiveness to pituitary extracts.

The animal is killed by a blow on the head, and at once both horns of the uterus are removed and placed in Locke's solution. One entire horn, freed from the broad ligament, ovary, and Fallopian tube is suspended in the bath of Locke's solution at 38° C. We have never seen the necessity of including the ovary and Fallopian tube, as some workers do, but have felt, on the contrary, that it might introduce an error or at least some difficulty in the interpretation of results owing to gradual stretching of the Fallopian tube during the

course of an experiment. Since we know of no special advantage in their use we have uniformly discarded them.

After complete relaxation, which takes from 15 to 30 minutes, the preparation is so adjusted as to write a base line with but slight spontaneous movements. With small uteri of young animals this is readily accomplished by the weight of the lever, or a small additional weight if necessary. We have found that the Harvard aluminum heart lever, with a magnification of approximately 4, serves the purpose very well. This gives a magnification on the tracing of such magnitude as to make it easy to detect small differences in dosage. The extracts to be tested are always prediluted with Locke's solution so that the dose added to the bath of 100 c. c. is about 0.5 c. c., and never exceeds 1.0 c. c. The dilutions are usually made of such strength as to require nearly equal volumes of standard and unknown to elicit equivalent reactions. Each dose is carefully measured with pipettes accurately graduated to 0.01 c. c. After an equivalent dose of the unknown is found for a given dose of the standard, the respective doses are then increased or decreased, or both, by 10 to 20 per cent. This not only confirms the earlier finding, but also gives assurance of the sensitivity of the uterine segment to small increases or decreases of dosage.

The following is the composition of Locke's solution used in this work:

Sodium chloride	9.00 gm.
Calcium chloride	0.24 gm.
Potassium chloride	0.42 gm.
Sodium bicarbonate	0.50 gm.
Dextrose	0.50 gm.
Glass redistilled water to make	1,000.00 c. c

Our Locke's fluid is freshly prepared each day from stock solutions of twentyfold concentration exclusive of the dextrose. The dextrose is weighed out each time as required.

Burn and Dale (*8*) recommend a small amount of magnesium chloride to be added to Locke's solution as a means of lessening the tendency to irregular spontaneous rhythm in the uterine muscle. Our method of using oxygen passing through a 2 per cent solution of sodium bicarbonate, as described above, has given us results so gratifying that we have seen no necessity for trying out extensively anything new. We have, however, tried, since the appearance of their paper, a few experiments with Locke's fluid containing the recommended amount of magnesium chloride (0.0005 per cent), and have found it of advantage in some experiments, though in others it seems to have so reduced the sensitivity of the uterine muscle to pituitary extracts that small differences in dosage did not elicit distinct differences in reaction. By our method the uterine muscle

shows very little tendency to irregular spontaneous rhythm, its tone is maintained at a uniform level for hours, and it preserves a high and fairly constant degree of sensitivity toward pituitary extracts.

THE STANDARD FOR THE BIO-ASSAY OF PITUITARY EXTRACTS.

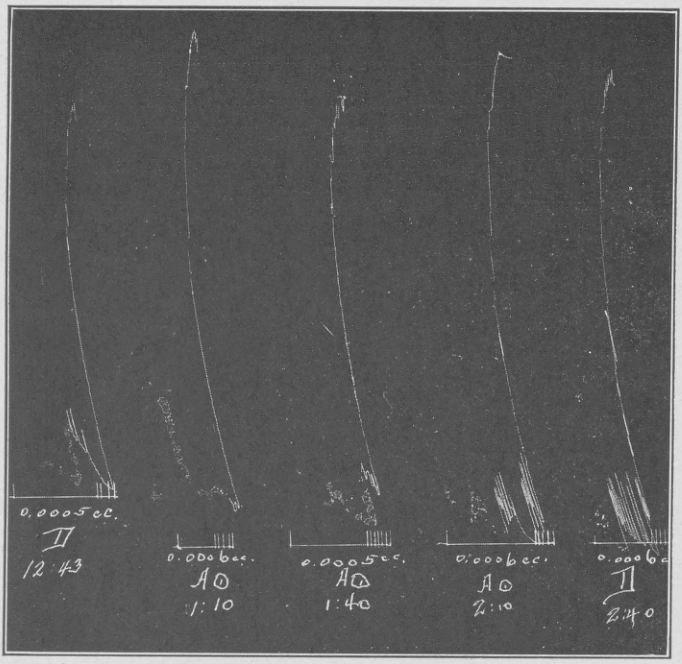
The great variations in activity of commercial extracts, and possibly other considerations must have led earlier workers (*2*) (*6*) to the assumption that preparations made from the infundibular lobe of the pituitary gland would lack uniformity and stability, and could not be used as a reliable standard for the assay of pituitary extracts. It is probably such considerations as these that led them to seek artificial standards. The unsatisfactory status of the artificial standards heretofore proposed, as pointed out earlier in this paper, led us to investigate the feasibility of using some preparation of the infundibular portion of the pituitary gland as a standard. Dale and Laidlaw, indeed, made this suggestion in 1912 (*9*). Obviously, none of the objections raised against artificial standards could be entertained against a preparation from the gland itself, provided, of course, it could be shown that such preparations possess all the activity of the gland, show uniformity in physiological activity, and remain stable over a reasonable length of time.

Since there appears to be some ground for the belief that rapid changes, perhaps autolytic, occur in the posterior lobes of pituitary glands subsequent to their removal we have invariably used fresh material. The glands were in all cases removed within 10 to 20 minutes of the killing of the animal and the posterior lobes were immediately dissected out and worked up according to the methods described below.

It appeared to us that an infundibular powder possessing the requirements as outlined above and yielding its entire activity upon simple extraction should be a most suitable standard for the assay of pituitary extracts. In order to determine the physiological activity of the infundibular powders made by us at various times, a simple acidulated saline extract of fresh infundibular lobes from the same batch was also prepared each time a lot of glands was gathered for the preparation of an infundibular powder. These extracts were made by weighing out accurately a definite amount of fresh posterior lobe material, which was then thoroughly ground to a fine pulp in an agate mortar with a little chemically pure sand, extracted with a measured volume of one-fourth of 1 per cent acetic acid in physiological salt solution, heated quickly to boiling and filtered. The filtrate was at once brought to the laboratory, put into ampoules, sterilized in steam at 100° C. on three successive days for 20 minutes each, and stored in the ice box. These fresh gland ex-

tracts were uniformly made to represent 5 per cent of fresh posterior lobe material.

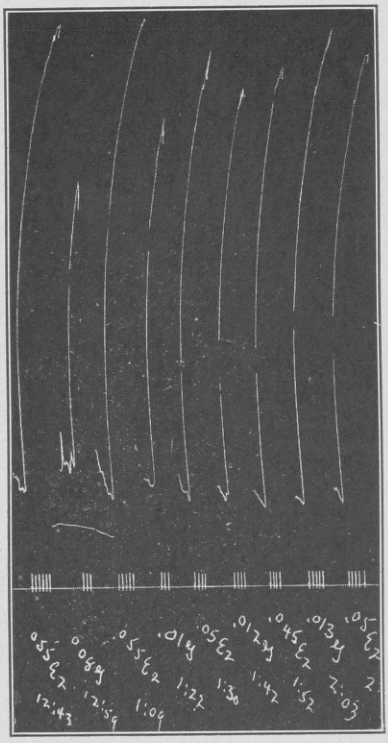
Primarily these extracts were made to serve as a standard of comparison of the activity of the respective infundibular powders. We have had occasion, however, to assay against each other eight such extracts made at different times during the months of June to October, 1922, and, contrary to our expectations, we were much impressed with the remarkable uniformity in their potency. Tracing



TRACING 1.—July 13, 1922. Guinea pig, 210 grams. Shows equality of 5 per cent fresh gland preparations A1 and D. In this, as in all subsequent tracings (except 5), the time is marked in minutes, and indicates the interval between adding the dose to the bath and replacing it with fresh Locke's solution.

1 shows the uniformity in activity of two such extracts, A 1 made in June and D prepared in July. We had, however, one notable exception to this. Five per cent fresh gland extract G, made by one of us in August with the same technic as the others showed only half the theoretical strength when assayed at the time of its preparation against extract D already referred to. Later the same extract was also assayed against one of our desiccated infundibular preparations E 2 and again was found to represent only 26.6 mg. fresh gland substance per c. c. (See tracing 2.) We are at a loss to explain this marked divergence.

Since the appearance of the recent publication of Burn and Dale (*8*) we have made some experiments to determine whether it would be possible to make 10 per cent extracts of uniform potency from fresh frozen posterior lobes by their technic.[3] A batch of fresh pituitary glands was obtained, which immediately upon removal were placed in a freezing mixture. They were brought in the frozen state to the laboratory, the posterior lobes carefully dissected out and minced

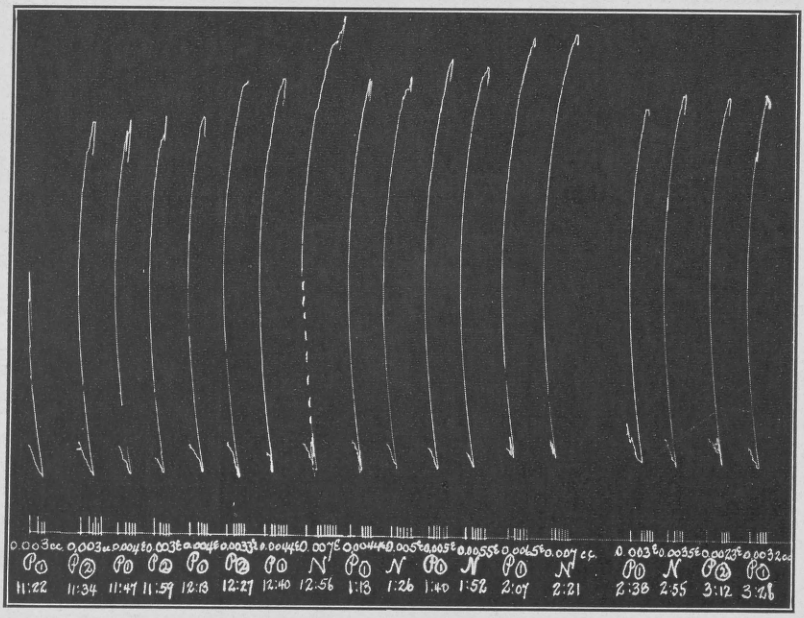


TRACING 2.—December 11, 1922. Uterus of guinea pig, 220 grams. Assay of 5 per cent fresh gland extract G against desiccated infundibular preparation E2. Result: 0.013 c. c. G = 0.05 mg. E2, or 1.0 c. c. G = 3.8 mgs. E2. Hence, 1.0 c. c. G = 3.8 × 7 = 26.6 mgs. fresh gland substance. (1 mg. powder = 7 mgs. fresh gland substance, as shown in Table I.)

finely with scissors. After mixing the whole mass of minced glands, two portions of 4.0 gm. each were weighed out, and two extracts made according to the details of the technic of Burn and Dale. The extracts were placed in ampoules and sterilized on three successive days for 20 minutes each in the Arnold sterilizer. These extracts,

[3] Burn and Dale made their extracts to represent 2.5 per cent of infundibular material. We made 10 per cent extracts in conformity with the recommendations of the subcommittee on bio-assay of the tenth revision of the United States Pharmacopœia. This however has undergone revision since the completion of these experiments, a 5 per cent extract having been substituted by the subcommittee.

designated P 1 and P 2, respectively, were later assayed against each other as well as against a 5 per cent fresh gland extract N, made by our method of extraction from the same material that P 1 and P 2 were made. The results of this assay are shown in tracing 3, from which it appears that the activity of extracts P 1 and P 2 is in the ratio of 3 : 4, the former representing the activity of 55 mg., and the latter 73.3 mg. of fresh gland substance per c. c. In a confirmatory assay of extract P 1 against one of our desiccated infun-



TRACING 3.—January 22, 1923. Guinea pig, 220 grams. P1 and P2 = 10 per cent extracts of minced fresh frozen posterior lobes of the pituitary, sterilized in the usual manner. N = 5 per cent extract of fresh frozen glands of same batch as above, made in the usual manner. Result: 0.003 c. c. P2 = 0.004 c. c. P1, and 0.005 c. c. P1 = 0.0055 c. c. N. Hence, 1 c. c. P1 = 1.1 c. c. N = 55.0 mgs. fresh gland substance, and 1 c. c. P2 = 55.0 × 4/3 = 73.3 mgs. fresh gland substance.

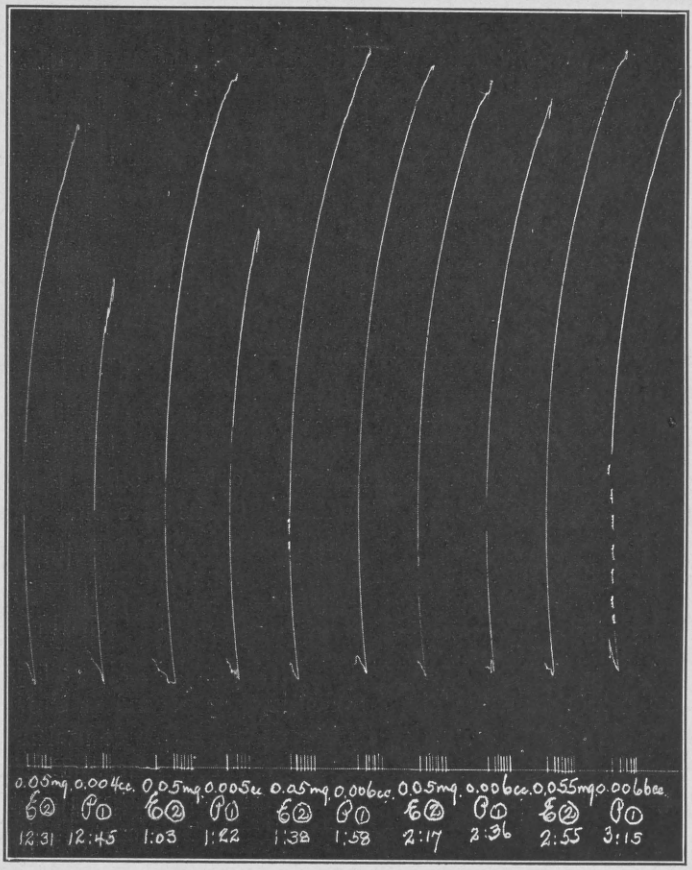
dibular powders E 2, one c. c. was found equal in activity to that of 8.3 mg. of the powder or 58.1 mg. fresh gland substance, since, as it will appear from Table I, approximately 7 mg. of fresh gland substance is the equivalent of 1 mg. of our powders. (See tracing 4.)

In the light of the work of Adams (*11*), to which reference will be made later, the possibility of sterilization affecting the activity of extracts P 1 and P 2 was considered, since their acidity is relatively low (about 0.07 per cent acetic acid [4]).

Another experiment was therefore carried out in which two extracts were made from the same batch of minced material. To the one acetic acid was added to make approximately 0.07 per cent and to the other

[4] As recommended by the subcommittee on revision. This has also been recently changed to 0.3 per cent glacial acetic acid.

sufficient to make 0.25 per cent. The two extracts were sterilized by fractional sterilization in the Arnold sterilizer and assayed against infundibular powder E 2. The former extract was found to represent the activity of about 40 mg. of fresh gland substance per c. c., and the latter 60 mg. Evidently low acidity, while a probable factor in reducing the potency of the extracts during frac-



TRACING 4.—January 19, 1923. Guinea pig, 240 grams. E2 = extract of desiccated infundibular preparation E2. P1 = 10 per cent extract of minced fresh frozen posterior lobes of the pituitary, sterilized in the usual manner. Result: 0.006 c. c. P1 = 0.05 mg. E2. Hence, 1 c. c. P1 = 8.3 mgs. E2 × 7 = 58.1 mgs. fresh gland substance.

tional sterilization, does not entirely account for the relatively low activity.

This serves to emphasize the point which Burn and Dale (*8*) stressed, viz, the great care that has to be practiced in the preparation of extracts from the fresh infundibular lobe of the pituitary gland, and the importance of adhering to strict details in their preparation, if they are to be made of uniform activity, the prime

requisite of a standard. In view of the great difficulty of making such extracts of uniform activity even with the best of technic and where conditions are most carefully controlled, it seems to us that a liquid " standard extract " is not an ideal standard for the bio-assay of commercial extracts.

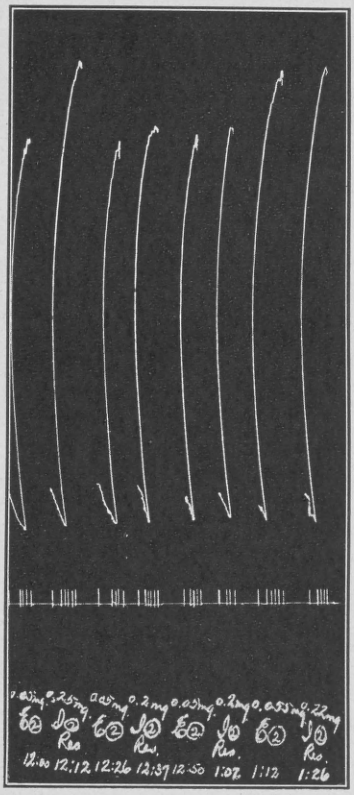
The infundibular powders which we have found to possess a high degree of uniformity in activity, which yield upon simple extraction the activity of the whole gland, and which have suffered no deterioration up to this time (a period of nearly nine months), were made in the following manner.

The carefully dissected fresh posterior lobes, about 40 in number, obtained within 10 to 20 minutes of the killing of the animal, are dropped at once into a flask containing about 150 c. c. of acetone.[5] Forty to sixty glands will suffice for 1.5 to 2 gm. of the powder. This treatment rapidly dehydrates the glands and stops all enzymatic action. The glands are then brought to the laboratory. At this time they are shrunken and present a tough leathery consistency. They are then cut up into small bits with scissors, placed in a fresh portion of acetone, and put away in the ice box. By the following morning the glands are pretty well dehydrated, considerably defatted, and have lost over two-thirds of their original weight. They are then dried in a vacuum desiccator over calcium chloride at a temperature not exceeding 50° C. After about five hours' drying the weight of the material is a little less than one-fifth of the original weight. The dried material is then ground in an agate mortar to a fine powder that will pass a No. 40 sieve. A small amount of residue remains that can not be reduced to the fine powder, but generally does not constitute much more than about 5 per cent of the finished product. The powder is then placed in the vacuum desiccator. The following day the powder is extracted with acetone in a small Soxhlet apparatus for about three hours. During this operation the loss in weight in one instance was 15 per cent of the weight of the powder prior to extraction. The extracted powder is then dried in vacuo over night at a temperature of about 40° C. to constant weight. The powder, after it is thus reduced to a constant weight, represents approximately 16 per cent of the original weight of the fresh glands. The finished powders are kept in the laboratory out of the light in a vacuum desiccator charged with calcium chloride.

The residue remaining after the material is reduced to a fine powder represents only part of the activity of the powder. This was determined for desiccated preparations H 2 (Table I) and I 2. In the latter instance 85 mg. of residue, or 6 per cent of the finished

[5] The fresh glands representing the powders described in Table I were weighed just before they were placed in the acetone.

product, were obtained. An extract of the entire residue was made, and this was assayed against an extract of desiccated infundibular preparation E 2. Inspection of tracing 5 shows that the residue represents only about one-fourth of the activity of the powder. Likewise the entire residue of desiccated infundibular preparation H 2 was extracted and assayed against our "standard extract D,"



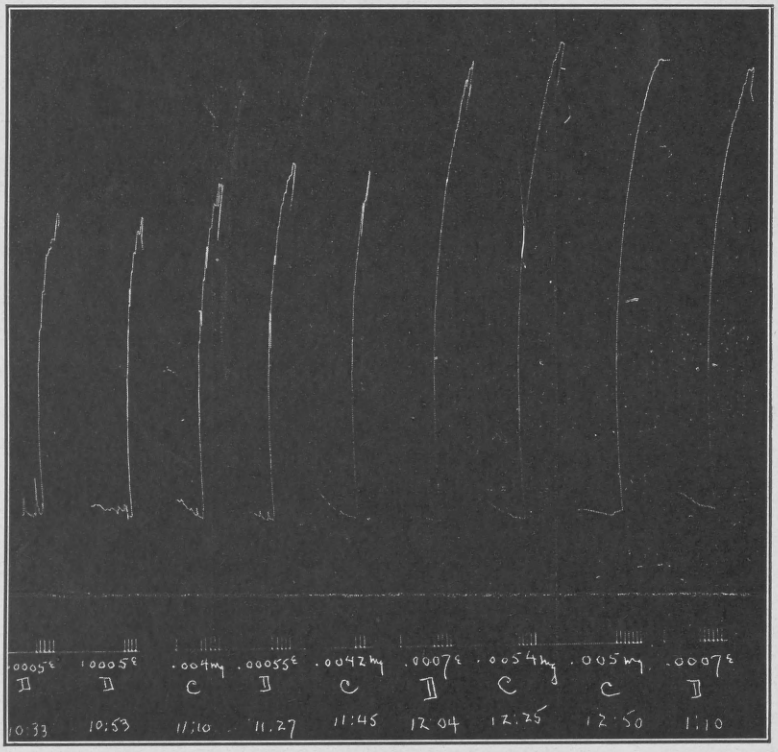
TRACING 5.—January 13, 1923. Guinea pig, 240 grams. Assay of residue obtained from desiccated infundibular preparation I2 against desiccated infundibular preparation E2. 0.2 mg. residue I2 = 0.05 mg. E2; hence, total residue of I2 (85 mgs.) = 21.2 mgs. of active powder.

to which we shall refer later, and it was found to represent about one-fifth of the activity of the corresponding powder.

At the beginning of the work it seemed desirable to have some definite uniform standard for the determination of the activity of the infundibular powders which we were planning to make from time to time. For this purpose 100 c. c. of a 5 per cent fresh gland extract were carefully prepared early in July, placed in ampoules, sterilized in the manner already described, and stored in the ice box. This we designated "standard extract D," and the activity of the six desic-

cated infundibular preparations made during the months of June to October (see table I) was determined and expressed in terms of milligrams of fresh gland substance represented in extract D.

The extracts of the desiccated and defatted infundibular preparations are made as follows. Exactly 10 mg. of the powder are weighed out accurately and transferred to an agate mortar. Exactly 10 c. c. of one-fourth of 1 per cent acetic acid in distilled water are measured



TRACING 6.—October 16, 1922. Uterus of guinea pig weighing 200 grams. Assay of desiccated infundibular preparation C against fresh gland extract D. Result: 0.0042 mg. C = 0.00055 c. c. D, and 0.005 mg. C = 0.0007 c. c. D; thus, 1 mg. C = 0.135 c. c. D. Hence, 1 mg. C = 0.135 × 50 = 6.8 mgs. fresh gland substance.

into a pyrex test tube of convenient size. The powder in the mortar is moistened with a drop or two of the solvent and is then thoroughly triturated to an impalpable frothy consistence. The remainder of the solvent is then added gradually and the mixture is thoroughly stirred for several minutes. This yields an opalescent solution, which is transferred back to the test tube, heated to boiling, and filtered. The clear filtrate thus represents the activity of 1 mg. of powder per cubic centimeter of solvent. Although we had invariably used this technic for extraction of the desiccated infundibu-

lar preparations, we were able to show subsequently that a much simpler method of extraction is equally efficacious. We shall refer to this again later.

The results of the assays as well as some other pertinent facts concerning the six desiccated and defatted infundibular preparations made during the months of June, July, August, and October are given in Table I. It will be remembered that the assays were made against 5 per cent fresh gland extract D. By way of illustration tracing 6 is appended showing one of the four experiments made to estimate the activity of infundibular powder C.

TABLE I.—*Potency of desiccated and defatted infundibular preparations of the pituitary gland.*

Preparation No.	Time of preparation 1922.	One mg. of powdered preparation = mg. of fresh gland.					Potency of preparation (average by assay / by actual weight).	Residue per cent of powdered preparation.
		By assay.				By actual weight.		
		Experiment No.	Mg.	Average.	Variation per cent.			
A 2	June	1	7.0			[1] 6.0	[1] 120	[2]
		2	7.0		+4			
		3	7.5	7.2	−1			
		4	7.1					
C	do	1	6.8			[2]		
		2	7.5		+4			
		3	6.9	7.2	−5			
		4	7.5					
E 2	July	1	5.7			6.3	107	5
		2	6.7		+23			
		3	6.3	6.7	−14			
		4	6.6					
		5	8.3					
F 2	do	1	6.7			6.2	108	7
		2	6.7		+8			
		3	6.3	6.7				
		4	6.7		−6			
		5	7.2					
G 2	August	1	8.3			7.4	101	16
		2	7.8					
		3	6.9	7.5	+10			
		4	7.5		−8			
		5	6.9					
H 2	October	1	7.4			6.3	118	8
		2	7.5	7.4	+2			
		3	7.1		−3			
		4	7.4					

[1] Approximately. [2] Not determined.

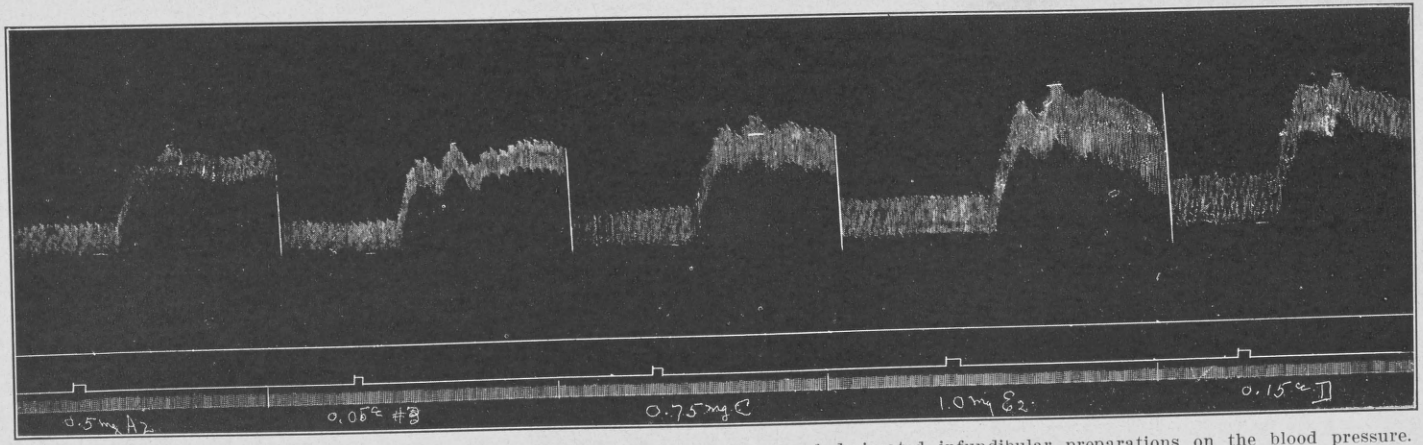
It will appear upon examination of Table I that though the variation of an individual assay of a given powder may be over 20 per cent, the average value of activity of 1 mg. of the several powders is approximately equivalent to that of 7.0 mg. of fresh gland substance, with a variation of less than 5 per cent from the average. It is further of interest to note that the activity of the powders by assay is somewhat higher than their value by actual weight. Thus 1 mg. of the desiccated and defatted powders represents a little over 6 mg. of fresh gland substance by actual weight, while by assay against

D, approximately 7 mg. The relatively high figure for powder G 2 (7.4 mg.) is due to the large amount of discarded residue which undoubtedly could have been reduced to the usual percentage by further grinding. The slight and constant disproportion of the value of the powders as obtained by assay and by actual weight gives them an activity of a little over 100 per cent of that of the whole gland. This we believe is due to the more thorough and complete extraction of the powder than is possible in the case of the fresh gland.

It is therefore evident from the figures given that fresh infundibular material, dehydrated, desiccated, and defatted as described yields powdered preparations of remarkably uniform potency, representing the entire activity of the whole gland, at least in so far as concerns the oxytocic principle.

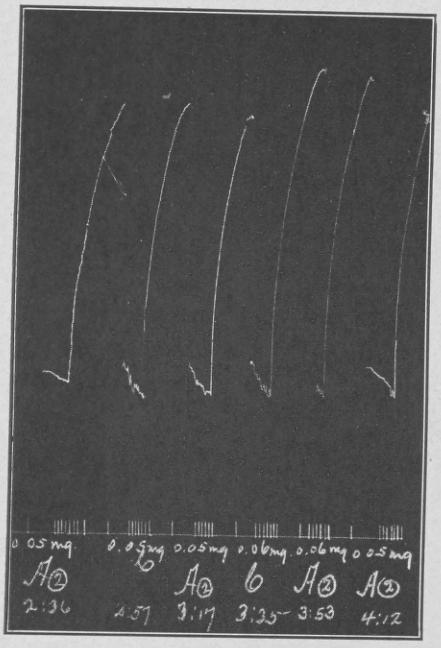
In order to further assure ourselves that our desiccated preparations represent the whole activity of the gland, several experiments were made to determine the activity of these preparations as regards the pressor principle. These experiments were carried out upon dogs under morphine and chloretone anaesthesia. Atropine was injected intravenously, to eliminate vagal effects. The extracts were injected into the femoral vein and the effect on the blood pressure recorded. By giving small doses of the extracts sufficiently far apart, i. e., at 15 to 25 minute intervals, it is possible in our limited experience to get a sufficiently large series of consistent reactions to give one a very fair idea of the relative pressor activity of two or more preparations. The blood pressure method, we admit, does not detect differences in the pressor activity with as fine a sensitiveness as the isolated uterus detects differences in the oxytocic activity. Our experiments, nevertheless, were sufficiently definite and the results clearly indicate a pressor activity for our desiccated infundibular preparations quite consistent with their oxytocic activity as determined by the isolated uterus method. Reference to tracing 7 will show that 1.0 mg. of powder E 2 is equal in pressor activity to 0.15 c. c. 5 per cent extract D or 7.5 mg. fresh gland substance. Similarly the reaction to 0.5 mg. powder A 2 is exactly equal to that of 0.05 c. c. commercial extract No. 3, thus making 1 mg. of the powder equivalent in its pressor action to 0.1 c. c. of commercial extract No. 3. This ratio is quite in keeping with that for their oxytocic activity as will be shown later (see Table II).

As a further check upon the uniformity in activity of the infundibular powders, numerous assays were made at different times against each other. The results of some of these experiments are illustrated by tracings 8 to 14. Tracing 8 shows that defatted infundibular powder A 2 is equal in oxytocic activity to that of C. These are our earliest preparations which were made in June, 1922. Trac-

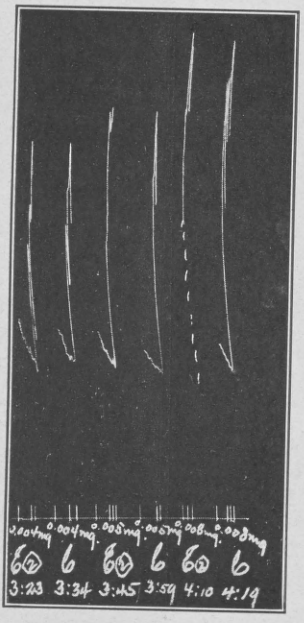


TRACING 7.—Dog, female, 6.2 kgs. morphine and chloretone. Effect of extracts of desiccated infundibular preparations on the blood pressure. The pressor action of 0.5 mg. A2 = that of 0.05 c. c. commercial preparation No. 3, thus making 1 c. c. of the latter equivalent to 10 mgs. of the infundibular preparation (cf. Chart 1). The reaction to 1.0 mg. of infundibular preparation E2 is equal to that of 0.15 c. c. D, hence the pressor action of 1.0 c. c. of fresh gland preparation D is equal to that of 6.7 mgs. of desiccated infundibular preparation E2 (cf. Chart 1).

ing 9 likewise shows no detectable difference in activity between infundibular powders C and E 2, the former having been made in June and the latter in July. Tracing 10 shows the result of an assay carried out in November upon infundibular powders F 2 and H 2. The former was made in July and the latter in October. The difference in activity, if any, is slight and insignificant, as it appears to be no greater than 5 per cent. Tracing 11 shows the result of an assay carried out in December upon infundibular powders E 2 and



TRACING 8.—Nov. 10, 1922. Guinea pig 170 grams. Shows equality of infundibular desiccated preparations A2 and C. Both prepared in June.

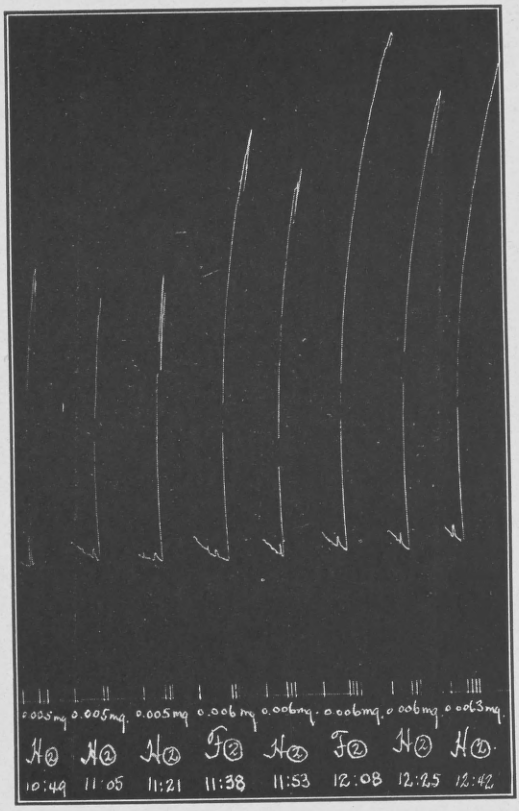


TRACING 9. — November 18, 1922. Guinea pig, 225 grams. Shows equality of infundibular desiccated preparations E2 and C. C was prepared in June, E2 in July.

G 2 made in July and August, respectively. These preparations are likewise shown to be equal in potency. The above tracings thus show equality in oxytocic activity of the desiccated infundibular preparations described in Table I, where they are also shown to be equal in activity when assayed against fresh gland extract D.

Since the completion of the work summarized in Table I another desiccated and defatted infundibular powder was made in the same manner as the others from glands gathered in December. This was designated I 2. This infundibular powder was assayed against

fresh gland extract D and the results indicated the same value for this powder as had been obtained for the others. This incidentally also showed that fresh gland extract D had not undergone any deterioration in five months. Tracings 12 and 13 show that infundibular powder I 2 made in December shows no appreciable difference in oxytocic activity from powders A 2 made in June and F 2 made in July.



TRACING 10.—November 22, 1922. Guinea pig, 190 grams. Shows approximate equality of infundibular desiccated preparations F2, made in July, and H2, made in October. The difference in potency of the two preparations does not exceed 5 per cent.

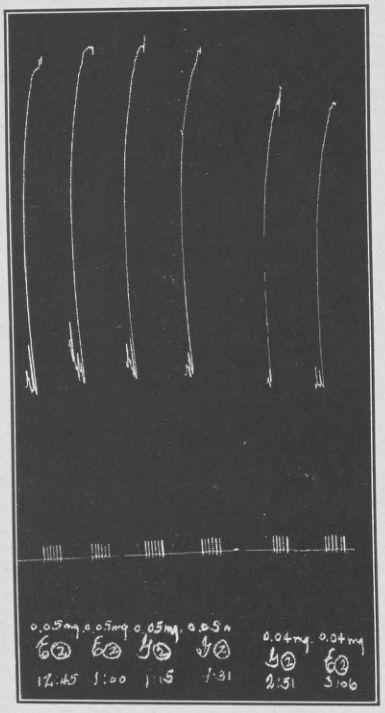
Another infundibular powder J 2 made recently in the same manner as the others from glands gathered in February and assayed against several of the earlier ones was likewise found to be of the same activity. Tracing 14 shows that powder J 2 made in February, 1923, is practically equal in activity to A 2 made in June, 1922.

The eight infundibular powders made during the months of June, 1922, to February, 1923, are thus shown to be uniform in activity, to represent the entire oxytocic and pressor activity of

the gland, to be free from seasonal variations in activity, in so far as our observations have gone, and to suffer no deterioration within a period of nearly nine months.

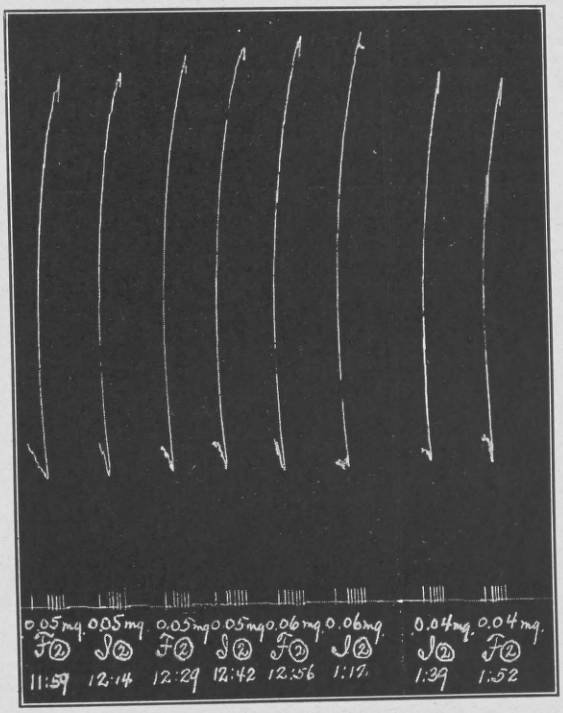
THE EXTRACTION OF THE DESICCATED AND DEFATTED INFUNDIBULAR PREPARATIONS.

During the major part of this work it was assumed that a very careful technic in the extraction should constitute an important factor in extracting all the activity of the infundibular powders.

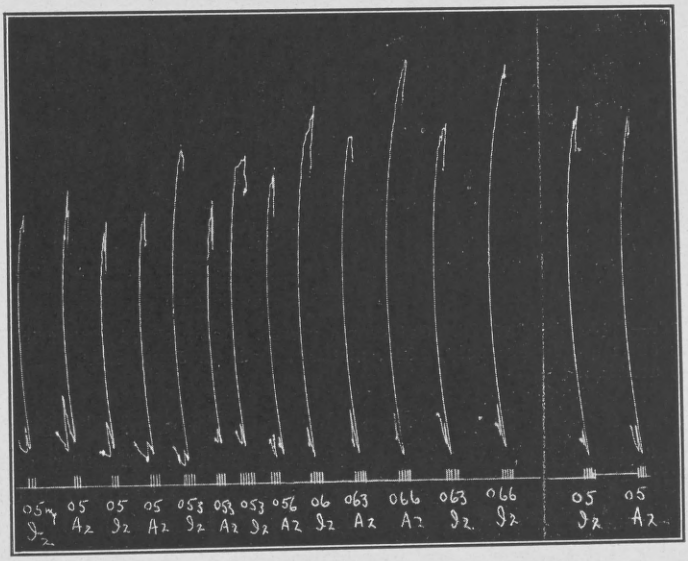


TRACING 11.—December 12, 1922. Guinea pig uterus. Shows equality of infundibular desiccated preparations E2 and G2. The former was prepared in July and the latter in August.

We have therefore uniformly employed the method of extraction previously described, the solvent being one-fourth of 1 per cent acetic acid in distilled water. Subsequently experiments were made to determine what factors if any are particularly concerned in the extraction of the oxytocic principle from our specially prepared powders. As a result of a series of tests it was found that simply vigorous shaking of the powder with distilled water in the proportion of 10 mg. of the powder to 10 c. c. of water for about 10 minutes,

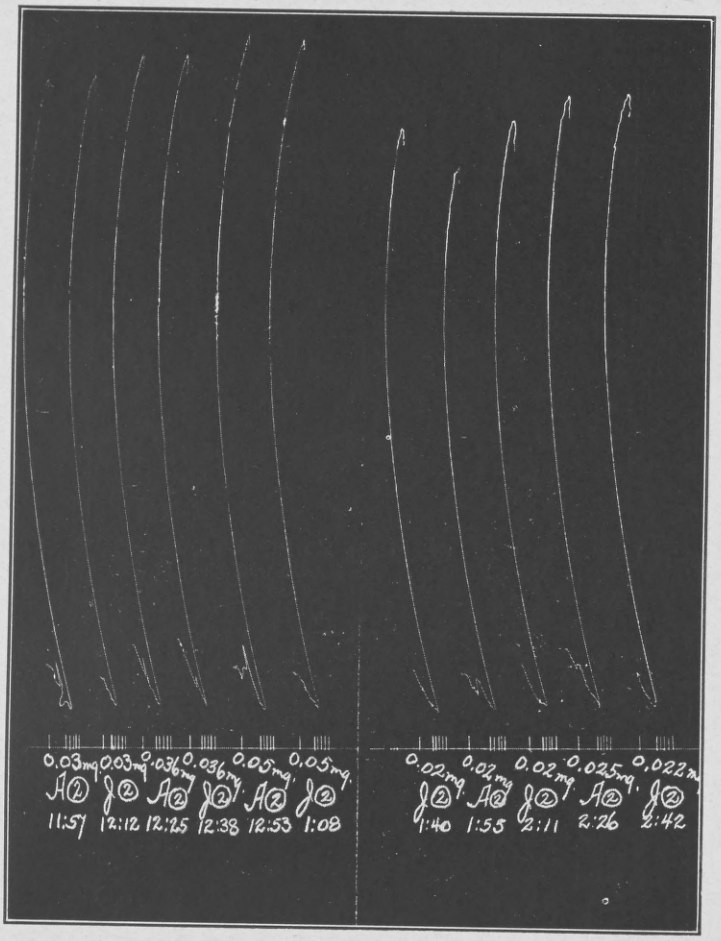


TRACING 12.—January 23, 1923. Guinea pig, 190 grams. Shows equality of desiccated infundibular preparations F2 and I2. The former was made in July and the latter in December, 1922.



TRACING 13.—December 16, 1922. Guinea pig, 200 grams. Shows equality of infundibular desiccated preparations A2 and I2. A2 was made from glands gathered in June; I2 was made from glands gathered in December.

then heating it quickly to boiling and filtering, will extract all of the oxytocic activity of the powder. Tracing 15 shows the result of such an experiment. One of the extracts of infundibular powder E 2 was made according to the rigorous technic we had generally been using, and the other extract was simply made by vigorously

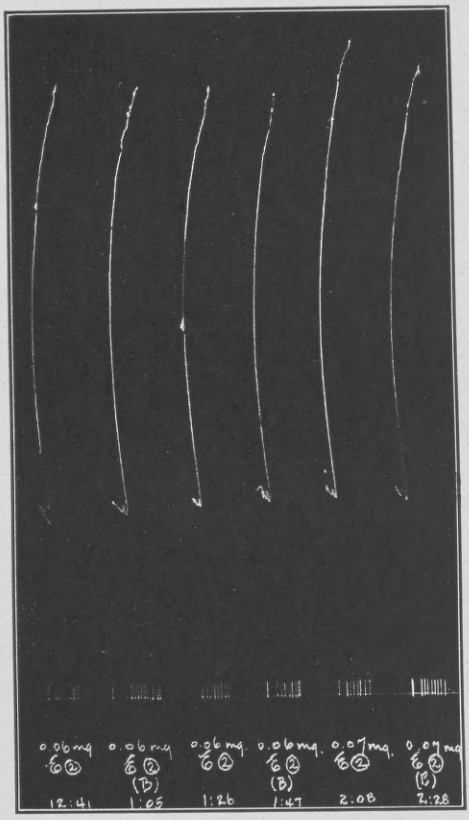


TRACING 14.—February 23, 1923. Guinea pig uterus. Assay showing that infundibular powders J2, made in February, 1923, and A2, made in June, 1922, are practically equal in activity.

shaking 10 mg. of the same powder with 10 c. c. distilled water in an accurately graduated glass-stoppered measuring cylinder, then boiling and filtering. The two extracts are shown to be equal.

Attention has been called by Burn and Dale (*8*) to the rapid deterioration in activity of fresh posterior lobes if kept for any length of time even at room temperature, and great stress is laid by them upon the minute details which must be practiced in the prepa-

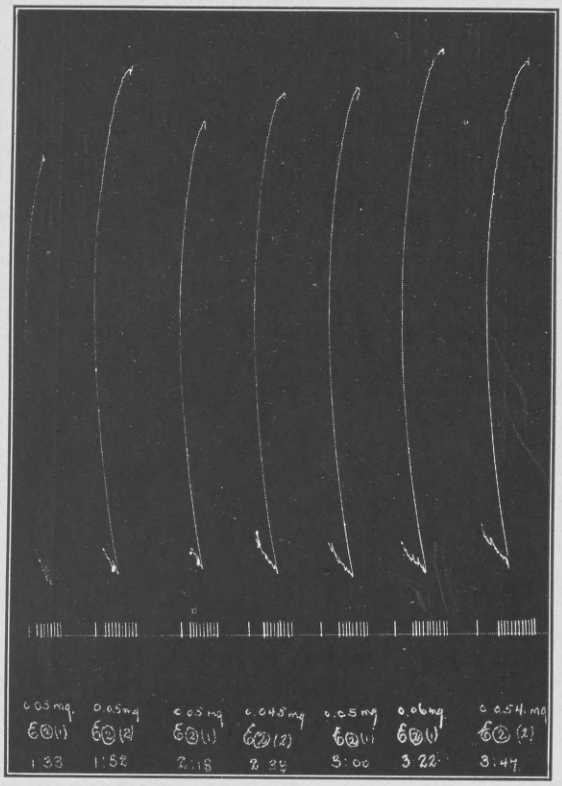
ration of extracts from fresh posterior lobes, in order to eliminate deterioration in activity from this cause. We have made some experiments to determine the effects of brief exposures at temperatures up to 38° C. on the activity of unboiled extracts of our infundibular powders. Extracts of the infundibular powders were prepared by our usual technic, but without boiling were left at room temperature



TRACING 15.—January 9, 1923. Guinea pig, 230 grams. Shows that vigorous shaking of desiccated infundibular preparations with distilled water for a few minutes will effect complete extraction of the oxytocic principle. E2, made in the usual manner (see text); E2 (B), made by shaking vigorously 10 mgs. of E2 powder with 10 c. c. distilled water for about 10 minutes, then boiled and filtered. Note the equality of the two extracts.

for 2 hours, or in the incubator at 38° C. for 2 and 28 hours, respectively. The extracts were then boiled, filtered and assayed against similar extracts made in the usual manner. No appreciable difference in activity was observed. Tracing 16 shows a difference of only 10 per cent between two extracts of powder E 2, the one made in the usual manner and the other incubated at 38° C. for 28 hours before boiling. The difference is in favor of the latter, but we are not inclined to attach much significance to a 10 per cent difference. No

difference in activity could be detected between freshly prepared extracts and those exposed to either room temperature or to incubator temperature for two hours before boiling. In our infundibular powders we thus have a preparation which requires no especially difficult technic for the extraction of its oxytocic principle. The matter of acidity of the solvent may of course be a very important factor if the extract is to be subjected to prolonged boiling, as in the



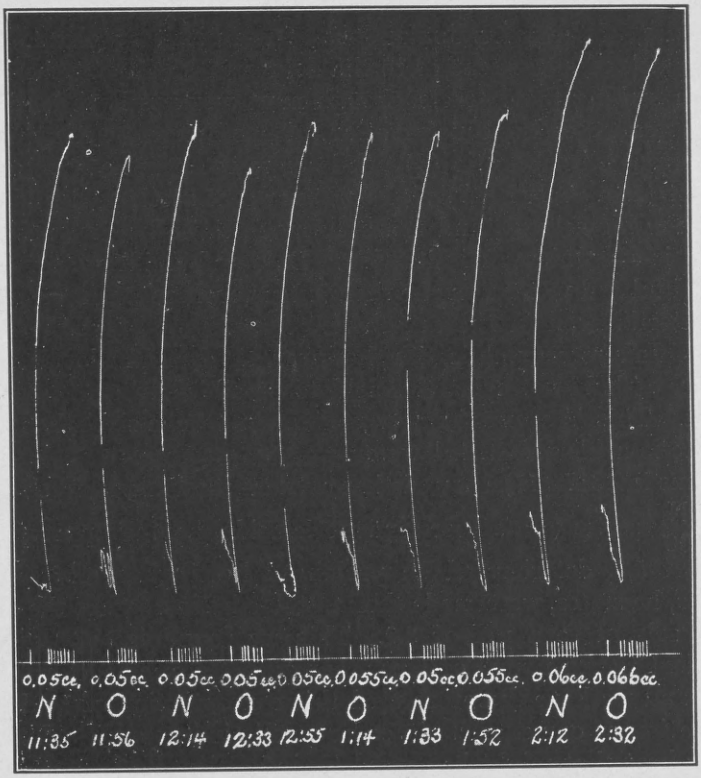
TRACING 16.—December 5, 1922. Guinea pig, 250 grams. Shows that incubation is not a factor in the extraction of the active principle from desiccated infundibular preparations. E2 (1), made in the usual manner (see text); E2 (2), extract incubated at 38° C. for 28 hours, then boiled and filtered. Note the approximate equality of the two extracts, the difference not exceeding 10 per cent.

process of sterilization. This was pointed out by Adams (*11*), and we shall refer to it later.

THE RELATIVE OXYTOCIC ACTIVITY OF POSTERIOR LOBES OF THE PITUITARY FROM STEERS AND FROM COWS.

The material obtained for our work at the local slaughterhouse has been of mixed stock, both steers and cows being represented. We understand that commercial extracts are generally made from glands

of steers. Because of this consideration in particular, apart from the general physiologic interest thereof, an experiment was made to determine whether there is any difference in the oxytocic activity of pituitary glands from males and females. The whole pituitary glands from steers and from cows were placed immediately following their removal in a freezing mixture, and in this condition were



TRACING 17.—January 18, 1923. Guinea pig, 210 grams. Comparison of activity of infundibular extracts made from posterior lobes of pituitary glands of steers and of cows. N, 5 per cent fresh gland extract of posterior lobes from steers; O, similar extract of posterior lobes from cows. Note the approximate equality in potency of the two preparations, the difference not exceeding 10 per cent.

brought to the laboratory. The posterior lobes of the respective batches were then carefully dissected out and immediately worked up into 5 per cent extracts and sterilized by the methods described earlier in the paper. We shall presently show that the oxytocic activity of fresh pituitary glands kept in a frozen condition for several hours is not altered from that of perfectly fresh glands. An assay carried out upon the two 5 per cent extracts against each other showed a difference of not more than 10 per cent. (Tracing 17.) We

conclude therefore that there is no appreciable difference in the oxytocic activity of pituitary glands of steers and cows.

THE EFFECT OF STERILIZATION ON THE OXYTOCIC ACTIVITY OF PITUITARY EXTRACTS.

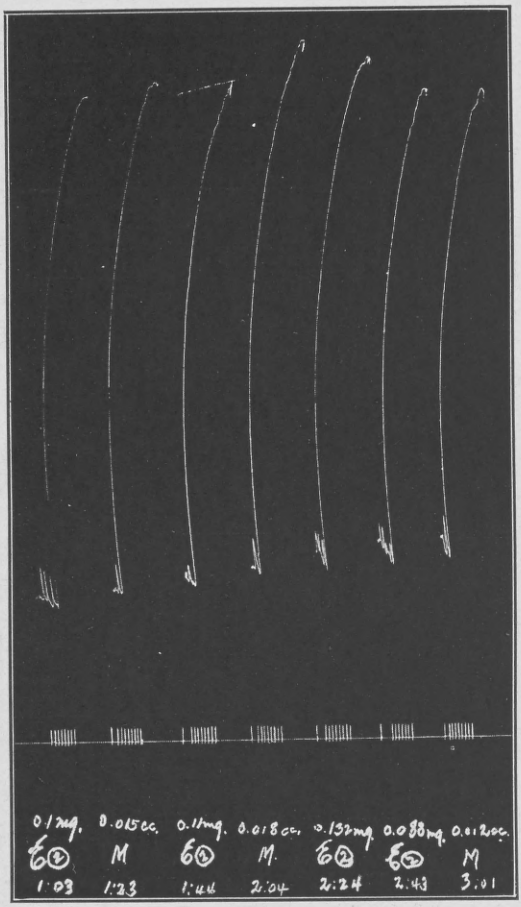
There appears to be no definite agreement as to the method of sterilizing pituitary extracts, nor is there definite information concerning the effects of sterilization upon the active principle, or principles, of the infundibular lobe of the pituitary gland. There is also lack of agreement as to the degree of acidity of the solvent used in making the extracts, though most workers have used acetic acid in greater or less concentration. Thus Fenger at one time used 0.5 per cent glacial acetic (*3*), while more recently he has used 0.25 per cent (*12*). Roth (*13*) used 0.005 per cent acetic acid in distilled water for his solvent, and the only significance he attaches to the acidity is convenience of filtration. Dale and Laidlaw in 1912 (*9*) used acidulated water to make their extracts which lost some activity upon sterilization in the autoclave. The degree of acidity is not stated. In a recent publication, however, Burn and Dale (*8*) specify 0.2 c. c. of half normal acetic acid to be added to each 40 c. c of solvent. This is approximately equivalent to one-sixtieth of 1 per cent acetic acid in distilled water.

Adams in 1917 (*11*) showed that extracts made from fresh glands with $\frac{N}{60}$ acetic (= 0.1 per cent acetic acid), having approximately a pH of 5.0, deteriorated on prolonged heating at 100° C. The extracts, he states, were made thermostable by increasing the acidity of the solvent to a pH of 3.0. This we found is approximately equivalent to one-fourth of 1 per cent glacial acetic acid in distilled water, and we have therefore used this concentration of acid in the preparation of our extracts in order to eliminate possible deterioration by heat that might be caused by insufficient acidity.

We examined the effects of two methods of sterilization upon the activity of pituitary extracts made with one-quarter of 1 per cent glacial acetic acid in distilled water or physiological salt solution as a solvent; (1) fractional sterilization on three successive days for 20 minutes each in the Arnold sterilizer, and (2) sterilization in the autoclave at 15 pounds pressure. The material was always put into ampoules and sealed. It should be noted that we had been using the former method of sterilization in our routine work, and from our observations had no reason to suspect any change in activity of the extracts from it. It seemed desirable, however, to make some carefully controlled experiments to determine this point. The experiments have shown that fractional sterilization had no effect upon the oxytocic activity of our extracts, while sterilization in the autoclave at 15 pounds pressure caused rapid deterioration of this prin-

ciple, the rate of deterioration being in proportion to the length of time of sterilization.

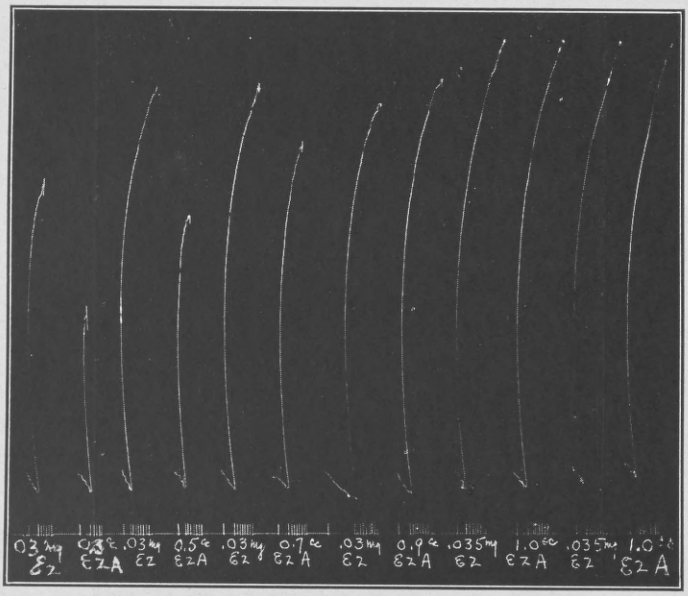
Tracing 18 is a tracing showing no effect on the oxytocic activity of an extract of infundibular powder E 2 made to represent 7 mg.



TRACING 18.—December 6, 1922. Guinea pig, 210 grams. Shows that the oxytocic principle can be extracted completely from desiccated infundibular preparations in concentration equivalent to 5 per cent fresh gland substance, and that steam sterilization on three successive days for 20 minutes does not affect the activity of such extracts. E2, extract of powder made in usual manner (see text); M, extract of E2, 7 mgs. per c. c. of one-fourth of 1 per cent acetic acid in distilled water, ampouled and sterilized in Arnold sterilizer on three successive days for 20 minutes. Result: 0.015 c. c. M = 0.11 mg. E2; also, 0.012 c. c. M = 0.088 mg. E2. Hence, 1 c. c. M = 7.3 mgs. E2.

of powder per c. c. of solvent (equivalent to a 5 per cent fresh gland extract) and sterilized by fractional sterilization, as assayed against an extract of the same powder made in the usual manner without sterilization. The results show no change in oxytocic activity.

Tracing 19 is presented to show the effect of sterilization in the autoclave on the oxytocic activity of pituitary extracts. An extract of infundibular powder E 2 was made in the usual manner. After filtration a portion was reserved for use as standard. The remainder was placed in ampoules, sealed, and sterilized in the autoclave at 15 pounds pressure for a period of 100 minutes. The assay showed that 65 per cent of the oxytocic activity of the sterilized extract was destroyed. A similar experiment showed that 15 minutes sterilization at 15 pounds pressure resulted in a loss of 25 per cent of oxytocic activity.



TRACING 19.—January 10, 1923. Guinea pig, 230 grams. Effect of autoclaving on the activity of infundibular extracts. E2, extract made from powder E2 in the usual manner; E2 (A), portion of same extract autoclaved at 15 pounds pressure for 100 minutes, and diluted 10 times. Result: 0.1 c. c. E2 (A) = 0.035 mg. E2, or 1.0 c. c. E2 (A) = 0.35 mg. E2. Hence, 65 per cent of the active oxytocic principle of E2 (A) extract was destroyed.

It may be concluded therefore that fractional sterilization at 100° C. does not affect the oxytocic principle of pituitary extracts made with one-fourth of 1 per cent glacial acetic acid as a solvent. Sterilization at 15 pounds pressure, even for a brief period, causes some deterioration in activity, the rate of deterioration rapidly increasing with increasing length of time in the autoclave.

THE ASSAY OF COMMERCIAL PITUITARY EXTRACTS, USING THE DESICCATED INFUNDIBULAR PREPARATIONS AS STANDARD.

Having found our infundibular preparations uniform in potency when assayed against each other as well as when assayed against

fresh gland standard extract D we next examined commercial extracts, using the infundibular powders as standard. Nine commercial extracts purchased in the open market, eight of which were of American make and one of British manufacture, were examined during the months of October and November, 1922. All the ampoules of each lot bore the same serial number, and we were later assured by the respective manufacturers that all ampoules bearing the same serial number had been filled with material from a single extract. Each of the extracts was assayed against each of our six infundibular powders described in Table I. The results of this investigation are summarized in Table II.

TABLE II.—*Assay of commercial pituitary extracts against the desiccated and defatted infundibular preparations.*

Column 1: Fraction of c. c. of commercial extract=1 mg. of desiccated infundibular preparation. Column 2: One c. c. of commercial extract=1 mg. fresh gland substance.]

Infundibular preparations.	Commercial pituitary extracts.																					
	1		2		3		4		5		6		7		8		9					
	c. c.	mg.	c. c.	mg.	c. c.	mg.	c. c.	mg.	c. c.	mg.	c. c.	mg.	c. c.	mg.	c. c.	mg.	c. c.	mg.	c. c.	mg.	c. c.	mg.
A 2	0.30 0.22	27.7	0.65	11.7	0.11	65.5	0.23	31.3	0.90	8.0	0.34	21.1	0.39	18.5	0.33	21.8	0.16	45.0				
C	0.33 0.25	24.8	0.50 0.75	11.6	0.10	72.0	0.23	31.3	0.89	8.1	0.45	16.0	0.35	20.6	0.37	19.5	0.18	40.0				
E 2	0.20 0.20	33.5	0.80 0.50	10.5	0.10	67.0	0.23	29.1	0.70	9.6	0.43	15.6	0.55	12.2	0.32	20.9	0.15	44.6				
F 2	0.17 0.20	36.2	0.60 0.60	11.3	0.10	67.0	0.20	33.5	0.70	9.6	0.40	16.7	0.48	13.9	0.38	17.7	0.19	35.2				
G 2	0.21 0.25	32.6	0.60 0.60	12.3	0.10	75.0	0.20	37.5	0.68	11.0	0.40	18.7	0.43	17.7	0.35	21.4	0.15	50.0				
H 2	0.20	37.0	0.68	10.9	0.11	67.3	0.23	32.0	0.90	8.2	0.36	20.5	0.45	16.4	0.29	25.5	0.16	46.2				
Average mg	----	31.9	----	11.4	----	68.9	----	32.4	----	9.1	----	18.1	----	16.6	----	21.1	----	43.5				
Variation per cent	---- ----	+16 −22	---- ----	+8 −8	---- ----	+8 −5	---- ----	+16 −10	---- ----	+21 −12	---- ----	+16 −14	---- ----	+24 −26	---- ----	+21 −16	---- ----	+15 −19				

The commercial extracts examined are numbered in the table from 1 to 9, as it seemed best to omit the names of the manufacturers from the present discussion. In the first column pertaining to each number, the fraction of a cubic centimeter of the commercial extract equivalent in potency to 1 mg. of the respective infundibular powders is given. In the second column pertaining to each number the value of 1 c. c. of the extract is expressed in terms of milligrams of fresh gland substance. These figures are arrived at by simple calculation, bearing in mind that 1 mg. of the infundibular powder is very nearly equal in oxytocic activity to 7 mg. of fresh infundibular substance. At the bottom of each column the average of the six or more assays in milligrams of fresh gland substance per cubic centimeter of extract is given, and the percentage variations of the individual assays from the average are indicated.

It will be seen that there is a wide variation in activity of the commercial extracts, ranging approximately from that of 9 mg. of fresh infundibular substance per cubic centimeter to nearly 70 mg.

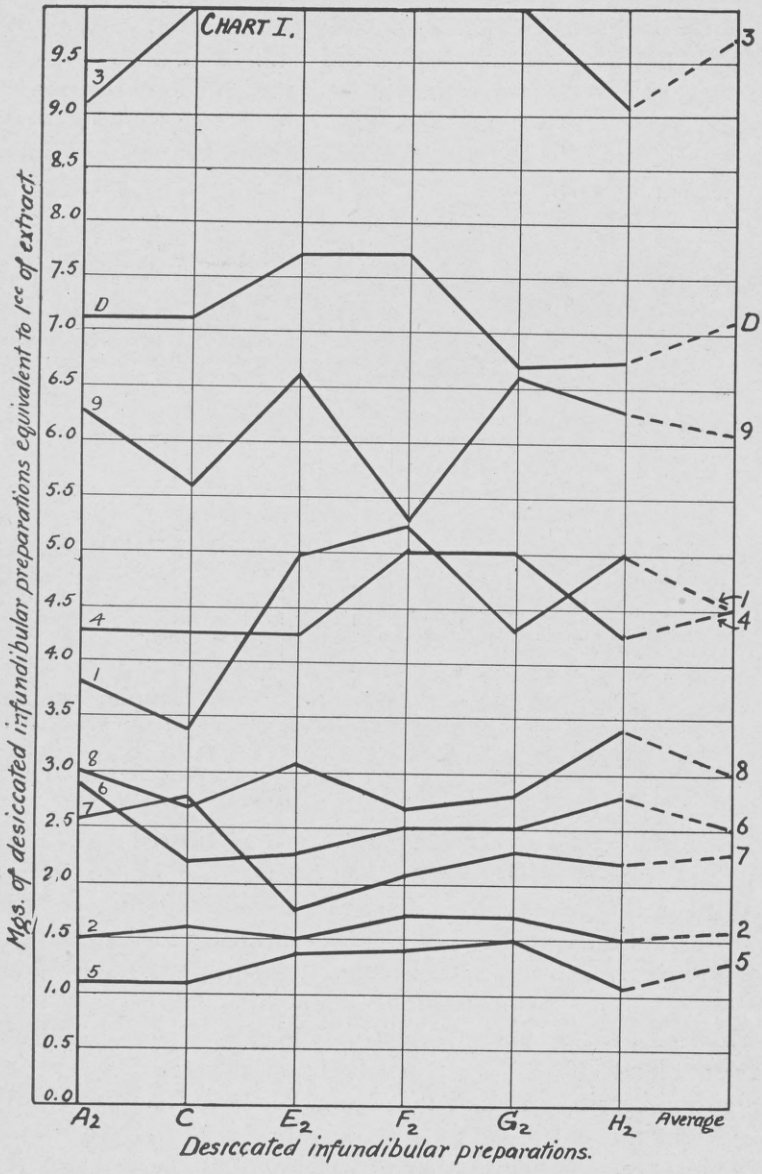


CHART I.—Potency of commercial pituitary extracts expressed in terms of milligrams of desiccated infundibular preparations equivalent to 1 cubic centimeter of extract.

It also appears that several of the extracts assayed at from 2 to 3 per cent of fresh infundibular substance, two at about 1 per cent, two at 4, and one at nearly 7 per cent.

The results summarized in Table II are plotted in Charts I and II. The curves in these charts are formed by joining the points on the scale giving the value of each assay in terms of milligrams of infundibular powder per cubic centimeter of extract as shown

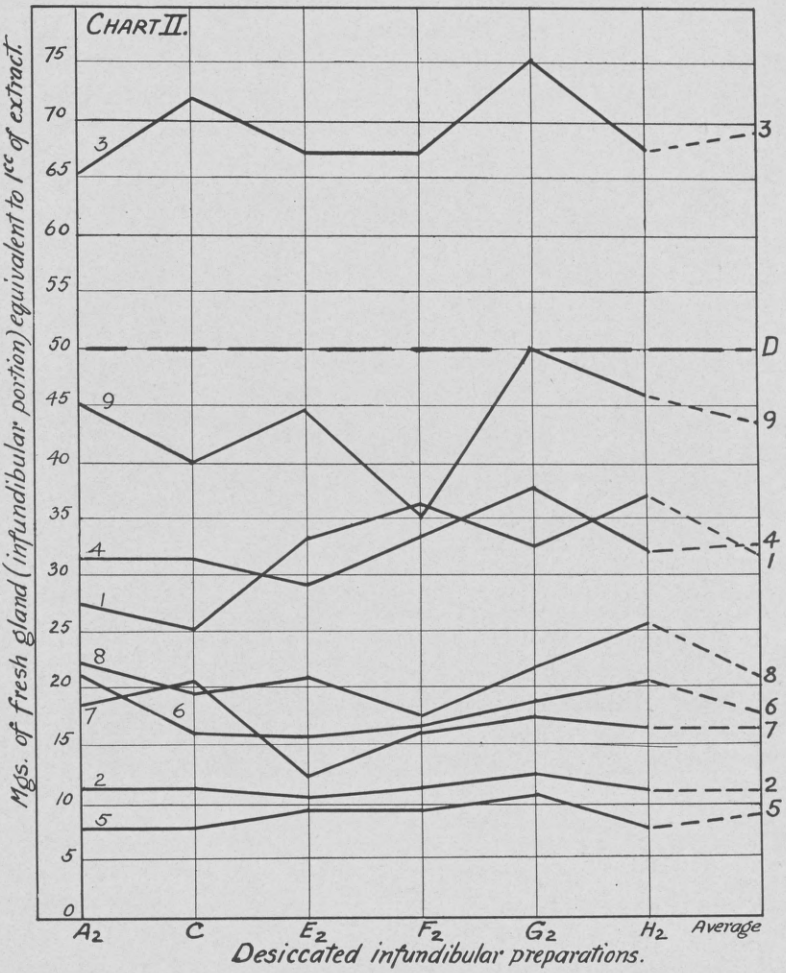
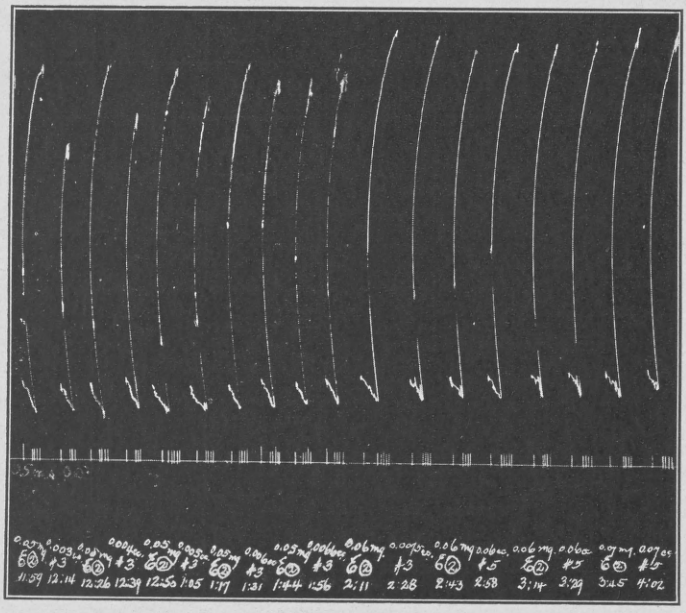


CHART II.—Activity of commercial pituitary extracts assayed against desiccated infundibular preparations, and potency expressed in terms of milligrams of fresh gland equivalent to 1 cubic centimeter of extract.

in Chart I, and in milligrams of fresh infundibular substance per cubic centimeter of extract in Chart II. The last point on the scale in each curve represents the average of the six or more assays. The curves are numbered to correspond to the numbers of the commercial extracts in Table II. Curve D in Chart I represents the value (individual assays and average) of our 5 per cent fresh gland

extract D in terms of activity of milligrams of infundibular powder per cubic centimeter.

As a further illustration of the wide variation in the activity of commercial extracts, tracing 20 shows assays of the weakest (No. 5) and the strongest (No. 3) extracts against infundibular powder E 2. It will appear from the tracing that 1 c. c. of commercial extract No. 3 is equivalent in activity to 8 mg. of the standard, while 1 c. c. of extract No. 5 represents the activity of only 1.0 mg. of the standard. Both commercial extracts assayed January, 1923, at some



TRACING 20.—January 12, 1923. Guinea pig, 240 grams. Assay of commercial extracts Nos. 3 and 5 against desiccated infundibular preparation E2. Result: 0.006 c. c. No. 3 = 0.05 mg. E2, and 0.0075 c. c. No. 3 = 0.06 mg. E2. Hence, 1 c. c. No. 3 = 8.0 mgs. E2, or 8 × 7 = 56 mgs. fresh gland substance. Also, 0.06 c. c. No. 5 = 0.06 mg. E2, and 0.07 c. c. No. 5 = 0.07 mg. E2. Hence, 1 c. c. No. 5 = 1.0 mg. E2, or 7.0 mgs. fresh gland substance; 1 c. c. of No. 3 is therefore equal in activity to 8.0 c. c. of No. 5.

20 per cent below the average found about three months previously, suggesting slight deterioration. The point of greatest interest, however, is the fact that the ratio of activity of the two extracts at this time of assay is 8, essentially the same as it was at the last assay three months previously; in other words, 1 c. c. of commercial extract No. 3 is equivalent in activity to 8 c. c. of commercial extract No. 5.

All commercial extracts examined were labeled "physiologically standardized," a term now obviously without meaning. Some of the preparations were also claimed to represent 20 and even 30 per cent of fresh posterior lobe material. From our experiments it appears

that the strongest preparation only represents 7 per cent of fresh infundibular material, while most of the others are considerably below our 5 per cent fresh gland extract D. Obviously, the manufacturers either succeeded in getting out only part of the activity of the gland, or lost much of it during the process of manufacture, or else the extracts suffered deterioration during the time intervening between their manufacture and the time of reaching us.

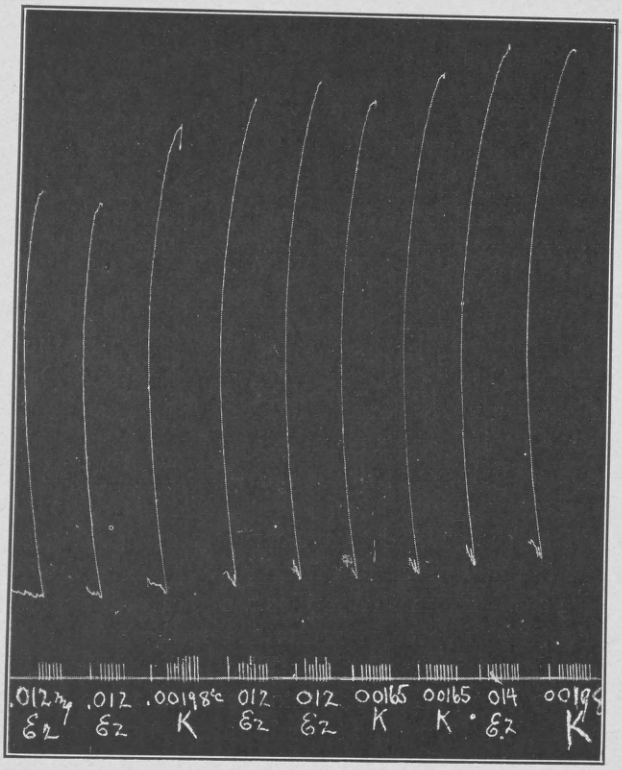
It was not surprising to find that the several commercial extracts vary in activity. Indeed, this was anticipated, since each manufacturer appears to be using his own standard, and there is lack of uniformity. It is difficult to understand, however, the great discrepancy between the activity of the extracts as assayed by us and as is often indicated on the label. To illustrate, two extracts that according to the manufacturers' statement were supposed to represent 20 per cent of fresh gland material, assayed at only a little over 3 and 4 per cent, respectively; and one, claimed to represent the activity of 30 gm. of fresh infundibular substance per 100 c. c. of solvent assayed at less than 1 per cent. It does not seem probable that the time element, within reasonable limits, would be a factor in the deterioration of extracts. Our 5 per cent fresh gland extract D, which was made in July, gave no evidence of deterioration at the time it was examined last, a period of six months.

In the hope of getting some information that might lead to the elimination of what appears to be so much waste, letters of inquiry were sent out to the several manufacturers concerning as much of the details of the manufacture of their products as they could conveniently disclose. From the replies we received we learned that, with the exception of three manufacturers who make their extracts from desiccated material, all the others make their extracts from frozen posterior lobes. The extracts were stated to represent anywhere from nearly 10 per cent to as high as 30 per cent of fresh infundibular substance. The age of the samples at the time of assay varied from 3 to 13 months. Four contained no preservative, while 5 contained some preservative. All were stated to have been sterilized, but the method of sterilization was only indicated in three instances. One of these extracts was stated to have been sterilized by heating in boiling water for 30 minutes, the other under steam pressure at 220° F., and the third by Berkefeld filtration. All were stated to have been physiologically standardized, though neither the method nor the standard were given, with one or two unimportant exceptions.

None of the information given us explained the relatively low activity of the preparations, as found by assay, in comparison with their theoretical strength. Indeed, some of the weakest preparations as

found by assay were stated to represent the highest percentage of infundibular substance.

Since most of the extracts, showing greatest discrepancy in their theoretical potency on the one hand and as found by assay on the other, were stated to have been made from frozen material it seemed possible though not probable that freezing the glands might affect their activity. An experiment was made to determine this point.



TRACING 21.—December 8, 1922. Guinea pig, 240 grams. Assay of 5 per cent extract of fresh frozen glands (K) against infundibular desiccated preparation E2. 0.00198 c. c. K = 0.014 mg. E2, or 1.0 c. c. K = 7.1 mgs. E2. Hence, 1.0 c. c. K = 7.1 × 7 = 49.7 mgs. fresh gland substance.

The whole pituitary glands were placed immediately upon removal in a freezing mixture, and brought into the laboratory in the frozen state. They were then placed in the cold room at a temperature varying from —10° to —18° C. The following day the posterior lobes were dissected out and worked up into a 5 per cent sterilized extract by the method previously described. Tracing 21 shows the result of an assay of this extract (designated K) against infundibular powder E 2. The assay indicated that 1 c. c. of this extract is equal in activity to 7.1 mg. of the powder, or 49.7 mg. of fresh

gland substance, since, as it will be recalled, 1 mg. of the powder is equivalent in activity to 7.0 mg. of fresh infundibular material. Clearly, this experiment indicates that freezing for several hours of the fresh pituitary gland does not affect its oxytocic activity. Freezing over a prolonged period of time might, of course, yield quite different results.

The reason for the poor yield of active principle in the commercial extracts is not altogether clear. However, simple as it is to make a watery or saline extract of the fresh posterior lobe of the pituitary, there are considerable difficulties in both the extraction and the purification, if it is desired to get out the active principles quantitatively. We have already alluded to this point earlier in the paper, and the recent work of Burn and Dale (*8*) has also emphasized it. While we are not conversant with the problems incidental to the manufacture on a large scale of commercial extracts from frozen material, nevertheless we can readily appreciate that the difficulties there would be far greater than those attending the preparation of an extract on a small scale in a well-equipped laboratory and under the best of technic. On the other hand, the extraction of the active principles from a stable and active infundibular powder, such as we have prepared, can be very easily made quantitatively with the ordinary amount of skill and care possessed by the average laboratory worker. We do not believe the preparation of such a powder on a large scale would be impracticable.

Now the question remains to be answered, what shall be the strength of the pituitary extract that the manufacturers shall be required to make and bring up by prescribed methods of assay to the set standard in order to insure uniformity in potency? Shall they be required to make an extract to correspond in potency to the weakest, such as numbers 2 and 5 or to the strongest as number 3 of the nine specimens we examined? This can not be answered definitely until carefully conducted clinical observations have been made using a definite laboratory unit of measuring potency as a basis. Theoretically, to prescribe one extreme may be as undesirable as to prescribe the other. The preparation which we have found to be the weakest may be ineffective in an emergency such as postpartum hemorrhage when administered in the accustomed dose; while on the other hand the usual dose of the strongest preparation may be altogether excessive for the ordinary therapeutic uses. The strength of a preparation is certainly not an index to its quality. Theoretically, at least, it should be just as easy to make a very strong extract as it is a very weak one, and from our experience we feel certain that this is true practically. Without definite clinical information, it is not possible to state at this time which would be most desirable for average use.

We believe that until such information is made available, which of necessity will have to be based upon cooperative work between the clinician and the laboratory worker, it would seem best at present to recommend that manufacturers make an extract 1.0 c. c. to represent the entire activity of 30 mg. of fresh infundibular substance, or approximately that of 4 mg. of our desiccated infundibular powder. This does not appear to be a very high requirement, should be easy to meet, and, we believe, would be a reasonably sound basis from which to start. Should it prove in time to be too strong or too weak for practical therapeutic uses, the manufacturers could then be asked by general agreement to increase or decrease its potency. This would of necessity eliminate the tendency on the part of some manufacturers to claim superiority for their product on the ground of its high potency, which is misleading and unjustifiable.

SUMMARY AND CONCLUSIONS.

The unsatisfactory status of the artificial standards heretofore proposed for the physiological assay of pituitary extracts is discussed.

A method is described for the preparation of an infundibular powder of uniform potency, representing the whole oxytocic and pressor activity of the infundibular lobe of the pituitary gland. The powder has not shown any deterioration within the period of observation of nearly nine months. The active principle or principles are readily extracted quantitatively from the powder by simple manipulation. The oxytocic activity of 1 mg. of this powder is equivalent to that of 7 mg. of fresh infundibular substance.

The results of the physiological assay of nine commercial extracts by the isolated uterus method, using the above powder as a standard, are given. It is shown that there is great variation in the activity of commercial extracts, the strongest having been found eight times as strong as the weakest.

It is proposed that an infundibular powder, such as is described herein, be used as a standard for the assay of commercial extracts by the isolated uterus method, and the recommendation is made that manufacturers be required to make and standardize their extracts to represent the activity of 4 mg. of the standard powder, or its approximate equal, which is 30 mg. of fresh gland substance per c. c. of extract.

It is further suggested that the recommended strength of the commercial extract remain in force until properly conducted clinical observations with carefully standardized extracts make a change desirable.

The following conclusions may also be drawn from the experiments described herein:

1. Infundibular lobes of the pituitary gland from cattle do not show seasonal variations in oxytocic activity.

2. There is no appreciable difference in oxytocic activity of infundibular lobes from steers and from cows.

3. Freezing freshly removed pituitary glands for several hours does not affect their oxytocic activity.

4. The oxytocic activity of properly acidulated extracts is not affected by fractional sterilization in steam at 100° C. Heating in the autoclave at 15 pounds pressure, for even brief periods, causes deterioration in activity.

REFERENCES.

(1) U. S. P. IX, Decennial Revision, p. 247.
(2) Roth, G. B.: Hyg. Lab. Bull., No. 100, 1914.
(3) Fenger, F.: J. Biol. Chem., 1916, v. 25 p. 417.
(4) Pittenger and Vanderkleed: J. Am. Pharm. Ass., 1917, v. 6, p. 131.
(5) Eckler, C. R.: Am. J. Pharm., 1917, v. 89, p. 195.
(6) Spaeth, R. A.: Hyg. Lab. Bull., No. 115, 1918.
(7) Nelson, E. E.: J. Pharmacol. & Exper. Therap., 1922, v. 19, p. 270
(8) Burn, J H., and Dale, H. H.: Med. Res. Counc. Spec. Rep. No. 69, 1922.
(9) Dale, H. H., and Laidlaw, P. P.: J. Pharmacol. & Exper. Therap., 1912–13, v. 4, p. 75.
(10) Trendelenburg, P., and Borgmann, E.: Biochem. Ztschr., 1920, v. 106, p. 239.
(11) Adams, H. S.: J. Biol. Chem., 1917, v. 30, p. 285.
(12) Fenger, F., and Hull, M.: J. Biol. Chem., 1920, v. 42, p. 152.
(13) Roth, G. B.: Hyg. Lab. Bull., No. 109, 1916, p. 26.

SOME FACTORS CONCERNED IN THE DETERIORATION OF PITUITARY EXTRACTS.[1]

By MAURICE I. SMITH, Pharmacologist, and W. T. MCCLOSKY, Assistant Pharmacologist, Hygienic Laboratory, Washington, D. C.

In a previous communication (*3*) we reported the results of our assay of several commercial pituitary extracts using as a standard a stable desiccated infundibular powder of uniform potency which was developed in this laboratory.[2] It was pointed out in this report that, aside from the great variation in activity of the commercial extracts examined, their potency, as determined by our assays against the standard powder and calculated in terms of equivalent amount of fresh infundibular substance, was much inferior to what one might expect from the figures indicated on the labels. Thus extracts, purporting to represent 10 to 30 per cent of fresh infundibular material, actually assayed at from 1 to 7 per cent. It would seem that the discrepancy could only be accounted for by one of two factors. First, the extracts may have lost much of their activity during the process of manufacture, or only part of the activity was obtained from the raw material. Second, the extract may have left the manufacturer in full theoretical strength, but deteriorated in activity through time, or other factors attendant upon storage.

Of the two problems at hand the latter is susceptible of solution, since all the factors concerned can be carefully controlled. The former does not readily yield itself to solution, since the method of preparation of the extract is somewhat of a secret with each manufacturer, and the conditons can not be accurately reproduced experimentally.

In this work an attempt has been made to determine the factors that might be concerned in the depreciation in the activity of an infundibular extract through the influence of time, temperature, and light. Some experiments were also made to determine under what conditions raw infundibular material will keep without loss of activity.

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[1] Manuscript submitted for publication October 6, 1923.

[2] Since our last report on the use of the infundibular powder as a standard for the bio-assay of pituitary extracts we have had occasion to prepare three more lots of such material in quantities of 2, 10, and 14 gm. each. These were made during the months of February, March, and August, respectively, and their activity was in nowise different from the seven samples previously reported on.

THE DETERIORATION OF INFUNDIBULAR EXTRACTS UNDER DIFFERENT CONDITIONS OF STORAGE.

The following experiments were performed: A 5 per cent acidulated aqueous extract of fresh infundibular material was prepared, distributed into ampoules and sterilized by fractional sterilization. This extract was designated D.[3] The greater bulk of this extract was placed in the ice box at a temperature of about 0° C. From time to time assays of this extract were made against the standard powder, by the isolated uterus method of Dale, to determine its oxytocic activity. The details of the method as carried out in this laboratory were described in the paper referred to (*3*).

Several ampoules of the same extract were placed in the incubator at 37° C., and this extract was likewise assayed at stated intervals, either against the standard powder or against the extract kept in the ice box. Similar assays were carried out at stated intervals upon some ampoules of the same extract kept at a constant temperature of 60° C.

In another series of experiments, quantitative acidulated aqueous extracts were made from the standard infundibular powder and designated M and K 2, respectively. The former was made of the strength of 7.0 mg. of standard powder per cubic centimeter, and the latter was made to contain the activity of 5.0 mg. of standard powder per cubic centimeter. The two extracts were placed in ampoules and sterilized by fractional sterilization at 100° C. Part of extract M was stored on a shelf in the laboratory in diffuse light, the remainder was placed in the incubator at 37° C. Extract K 2 was divided into three lots and stored at 37° C, 45° C., and 60° C., respectively. Assays of these extracts were made from time to time to determine their oxytocic activity, and in many instances experiments were also carried out upon the blood pressure of a dog with a view to ascertaining the effect of storage under different conditions on the pressor activity of the extracts. These experiments were intended not only as a confirmation of the findings by the isolated uterus method, but also as some contributory evidence on the question of identity or nonidentity of the oxytocic and pressor principle or principles in infundibular extracts.

The results of the observations are summarized in Table I. The assays were usually carried out in duplicate, and the figures recorded represent the averages.

[3] The method of preparation of this extract was described in the previous publication (*3*), in which experiments are also detailed showing the ratio of activity of this extract and the standard desiccated powder.

TABLE I.—*Effect of storage at different temperatures on the activity of infundibular extracts.*

Extract No.	Activity in mg. of standard powder.	Age at time of assay.	Activity at time of assay, mg. of standard powder.	Deterioration per cent.
ICE BOX TEMPERATURE (ABOUT 0° C.).				
D	7.0	1 year	7.0	0
LABORATORY TEMPERATURE. DIFFUSE SUNLIGHT.				
M	7.0	4 months	6.5	[3]
		5 months	6.3	
		6 months	6.7	
		8 months	[2] 6.5	
INCUBATOR TEMPERATURE (37° C.).				
D	7.0	1½ months	[1] 7.0	0
		2 months	[1] 7.0	0
		4 months	6.7	0
		6 months	[1, 2] 7.0	0
M	7.0	3 months	[1] 7.0	0
K2	5.0	1 month	5.0	0
		2 months	4.7	6
		3 months	[2] 3.9	22
INCUBATOR TEMPERATURE (45° C.).				
K2	5.0	1 week	[2] 3.9	22
		2 weeks	[2] 4.1	18
		1 month	3.8	24
		2 months	4.0	20
		3 months	3.6	28
INCUBATOR TEMPERATURE (60° C.).				
D	7.0	1½ months	[2] 1.3	82
K2	5.0	3 months	[2] 0.2	96

[1] Assayed against same extract kept in the ice box.
[2] Assayed also for its pressor activity by the blood-pressure method.
[3] Very slight if any deterioration.

Infundibular extract D was prepared in July, 1922, was standardized monthly for one year, and only the last assay, made in July, 1923, is indicated in the table, which shows it to have retained its entire activity. (See tracing 1.)

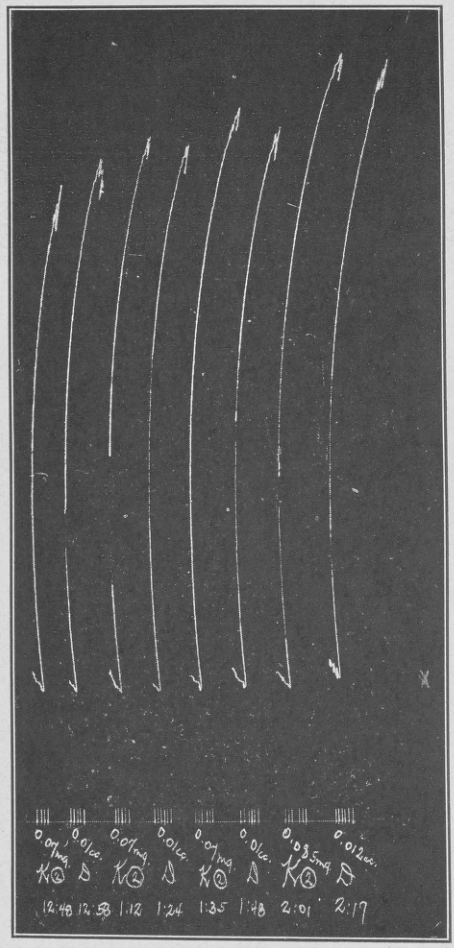
At no time during the year has a notable deviation been observed.

Extract D, kept in the incubator at 37° C. also failed to show any decrease in activity over a period of six months, when tested against the same extract kept in the ice box or against the standard infundibular powder. Similarly, extract M, made from the standard powder, retained its entire activity at 37° C. for 3 months.[4] The same ex-

[4] Tate(4) also has recently found that infundibular extracts of acidity ranging from 0.2 per cent to 2 per cent acetic acid lost none of their oxytocic activity on exposure to a temperature of 38° C. for 25 days.

tract, placed in ampoules and kept on a shelf in the laboratory in diffuse sunlight, retained practically its entire activity over an observation period of eight months.

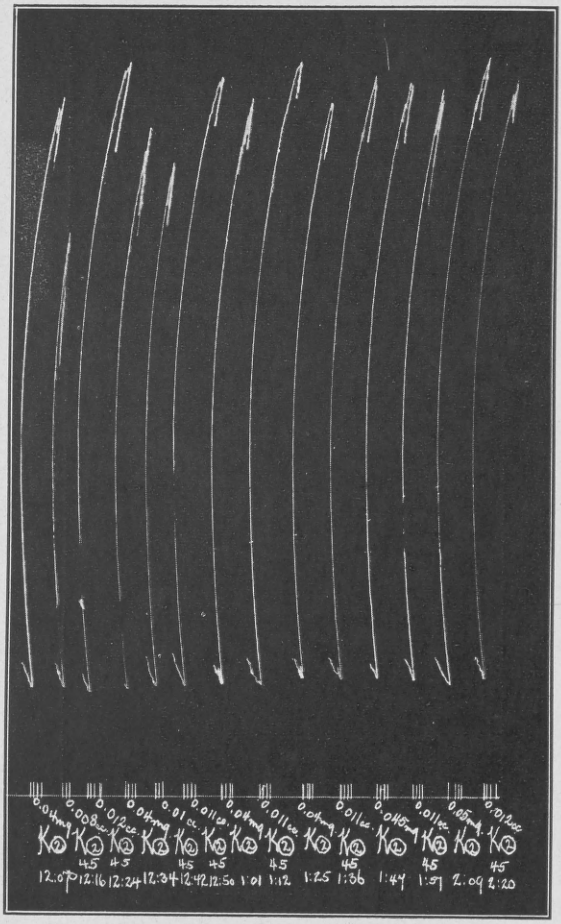
Extract K 2, made in the usual manner from the standard powder, lost very little of its activity at incubator temperature 45° C. The



TRACING 1.—July 12, 1923. Uterus of guinea pig weighing 210 grams. Assay showing no deterioration in oxytocic activity of extract D, which had been kept one year in the ice box (about 0° C.). Result: 0.01 c. c. D = 0.07 mg. K2 (standard powder). Hence, 1 c. c. D = 7.0 mgs. standard powder. (Time in minutes.)

extract remained perfectly clear throughout the period of observation of three months. This finding was so unexpected and interesting that a supplementary assay of the extract for its pressor activity seemed desirable. Tracings 2 and 3 indeed show close conformity of the oxytocic and pressor activity of this extract after remaining

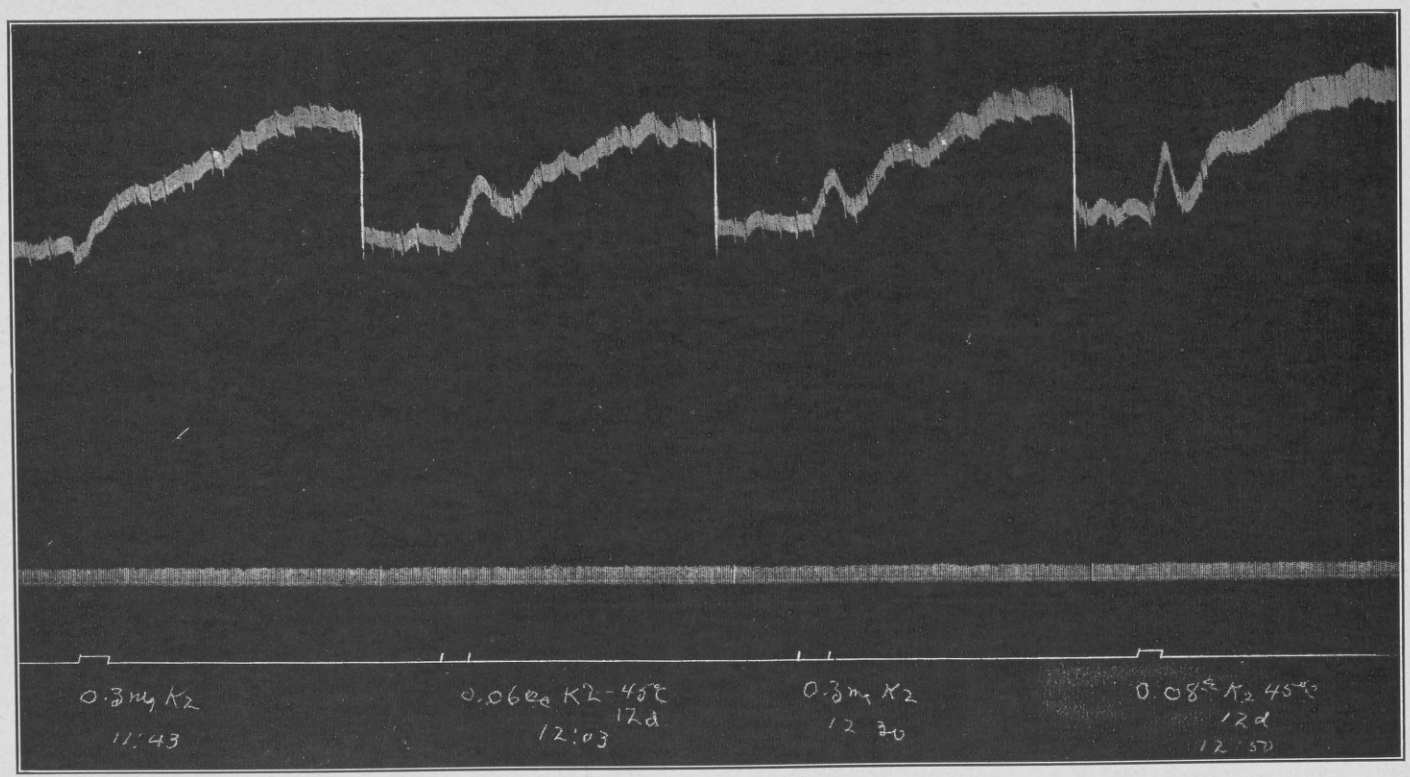
for one to two weeks at a temperature of 45° C. The loss in activity at this time was slight, and seemed to remain at about the same level for the entire period of observation of three months. It should be added that this extract, like all the others, was carefully standardized immediately after it was prepared and sterilized, and was



TRACING 2.—June 5, 1923. Uterus of guinea pig weighing 200 grams. Assay of extract K2 kept one week at 45° C. (K2–45) against standard infundibular powder K2. Result: 0.011 c. c. K2–45 = 0.045 mg. K2. Hence, 1 c. c. extract K2–45 = 4.1 mgs. standard powder, or 18 per cent deterioration. (Time in minutes.)

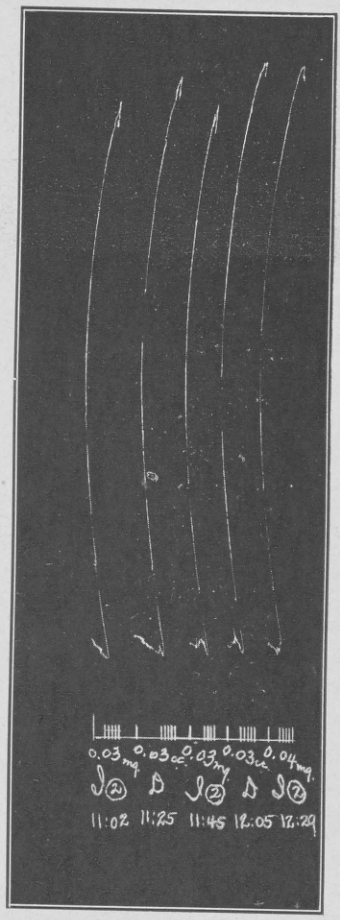
found to have the full theoretical activity, viz, 5 mg. of standard powder per cubic centimeter.

When kept at more elevated temperatures, the extract gradually loses its activity; both pressor and oxytocic activity apparently deteriorating at the same rate, as far as can be judged from the few observations. Extract D, kept in the incubator one and one-half



TRACING 3.—Dog, female; weight 8 kilos.: morphine and chloretone anesthesia; 10 mgs. atropine intravenously; time in seconds. Assay of the pressor activity of extract K–2 kept at 45° C. for 12 days (K2–45) against standard powder K2. Shows that 0.08 c. c. K2–45 is approximately equal to 0.3 mg. standard powder K2, making 1 c. c. of the extract = 3.8 mgs. K2 powder, or about 24 per cent deterioration in pressor activity (cf. Tracing No. 2).

months at 60° C., lost 80 per cent of its oxytocic activity, as shown in tracing 4; and, as nearly as could be determined, the same loss was found in its pressor activity. Extract K 2, kept under the same conditions for three months, retained only a trace of its oxytocic

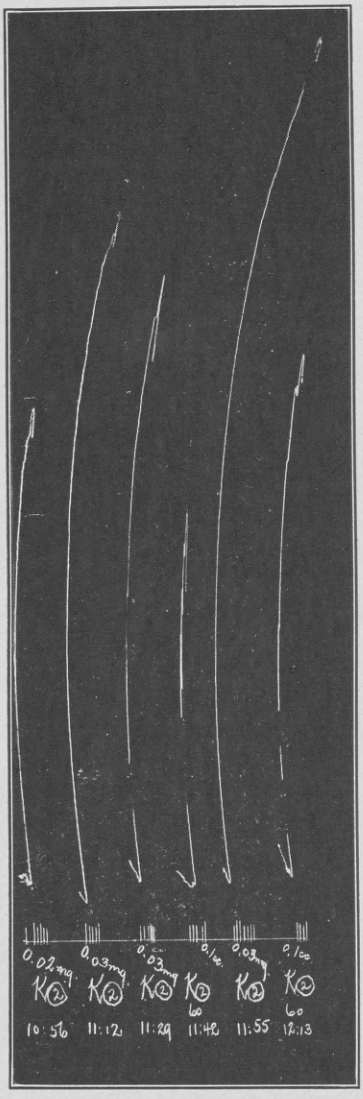


TRACING 4.—March 21, 1923. Uterus of guinea pig weighing 180 grams. Assay showing marked deterioration in oxytocic activity of extract D kept at a temperature of 60° C. for one and one-half months. Result: 0.03 c. c. D = 0.04 mg. standard powder 12. Hence, 1 c. c. D = 1.3 mgs. standard powder 12 or extract D deteriorated to the extent of $\frac{(7.0 - 1.3) \times 100}{7} = 82$ per cent. (Time in minutes.)

activity, viz, less than 6 per cent; and an indeterminable trace in pressor activity was likewise found by the blood pressure test, as is shown in tracings 5 and 6.

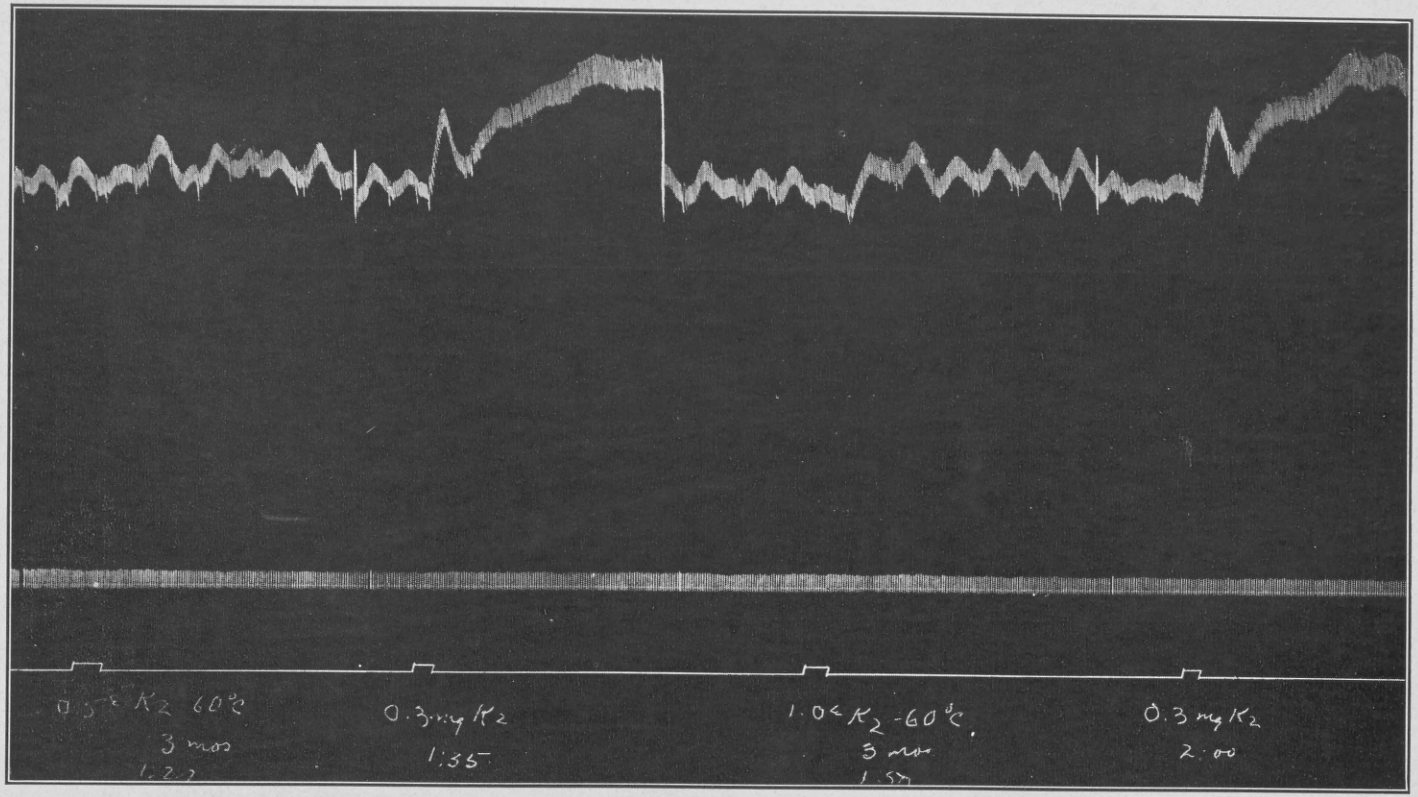
From a practical standpoint, the effect of the higher temperatures on prolonged storage need not concern us, for these conditions probably never prevail. Practically, it need only be emphasized that a

carefully prepared extract will retain its entire activity for at least a year, if kept in the cold; and further, will suffer no appreciable loss in either oxytocic or pressor activity over fairly long periods



TRACING 5.—June 27, 1923. Uterus of guinea pig weighing 250 grams. Assay showing nearly complete destruction of oxytocic principle in extract K2 kept at 60° C. (K2–60) over a period of three months. Result: 0.1 c. c. K2–60 less than and approximately equal to 0.02 mg. standard powder K2, or 1 c. c. = 0.2 mg. K2, thus showing about 96 per cent deterioration. (Time in minutes.)

of time, if exposed to such temperatures as may prevail during the shipment of the product. Neither does diffuse daylight at room temperature have a deteriorating effect on the extract in ampoules.



TRACING 6.—Dog, female; weight, 8.0 kilos.; morphine and chloretone anesthesia; atropine intravenously. Time in seconds. Shows nearly complete destruction of pressor principle in extract K2 kept at 60° C. over a period of three months. Note that 1 c. c. extract K2–60 shows much less activity than 0.3 mg. standard powder. The extract has thus retained only a trace of its original pressor activity. (Cf. Tracing 5.)

Furthermore, our observations lead us to question the necessity or even the wisdom of using a preservative, as some manufacturers do, provided the extract is made from good material and is carefully sterilized.

The effects of higher temperatures on the activity of infundibular extract are recorded here as information added to our rather meager knowledge of the chemistry of its active principle. The temperature at which marked destruction of the active principle occurs, under the conditions of the experiment, lies within the narrow limits of 45 and 60° C. The material in ampoules kept at 60° C. developed a marked turbidity, while that kept at 45° C. and below remained clear throughout. The gradual and nearly complete destruction of activity at 60° C. reminds us of Abel and Nagayama's observation, that boiling the extract with 0.5 per cent to 1 per cent HCl causes rapid and complete destruction of the pressor and oxytocic activity by hydrolysis (*1*). It is interesting that a similar process can occur at a much lower temperature and in a medium of 0.25 per cent acetic acid. The process does not appear to be the same, however, for Abel and Nagayama found depressor substances of the histamine type among the end products of hydrolysis; while we could detect no such substances in extracts D and K 2 after exposure for three months to a temperature of 60° C., and at a time when practically all of the oxytocic and pressor activity had disappeared.

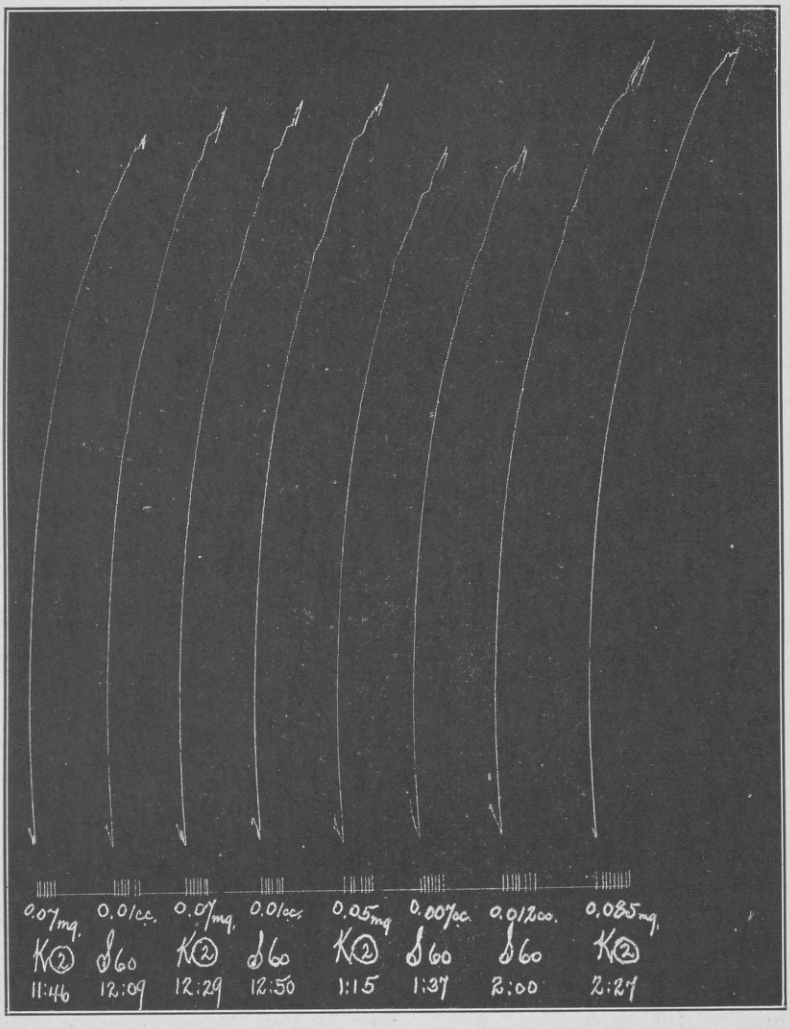
Furthermore, tests carried out for the biuret and Pauly reactions upon extracts D and K 2, at a time when they had practically completely deteriorated in oxytocic and pressor activity, yielded positive reactions and of an intensity indistinguishable from that given by the same extracts kept in the ice box and which had retained their entire activity. Abel and Nagayama, on the other hand, state that their purified extract failed to give the biuret reaction after hydrolysis, though the Pauly reaction remained unaltered.

The observation recorded here, that the oxytocic and pressor activity deteriorate in a quantitatively parallel manner, lends support to the conclusions of Abel and Rouiller (*2*) concerning the chemical identity of the oxytocic and pressor principle in infundibular extracts.

THE EFFECT OF STORAGE OF FRESH INFUNDIBULAR LOBES ON THE ACTIVITY OF EXTRACTS MADE THEREFROM.

The commercial manufacturer of pituitary extracts frequently employs material that has been in cold storage for a variable length of time. Having already shown (*3*) that freezing of whole pitui-

tary glands for several hours has no effect on the oxytocic activity of the infundibular extract made from such glands, it seemed desir-



TRACING 7.—September 24, 1923. Uterus of guinea pig weighing 240 grams. S60 = 5 per cent extract of frozen fresh infundibular lobes that had been kept for two months at a temperature of −10° C. to −17° C. K2, standard infundibular powder. Result: 0.01 c. c. S60 = 0.07 mg. K2; 0.007 c. c. S60 = 0.05 mg. K2; 0.012 c. c. S60 = 0.085 mg. K2. Hence, 1.0 c. c. S60 = 7.0 mgs. K2. (Time in minutes.)

able to ascertain how long chilled glands may be kept without deteriorating in activity.

The following experiment was performed: The whole pituitary glands of cattle were removed shortly after slaughtering and im-

mediately placed in a freezing mixture. The frozen glands were then brought to the laboratory, the infundibular lobes quickly and carefully dissected out, and the material divided into two lots. One lot was placed in the cold room with a temperature ranging from −2° C. to +2° C., while the other lot was placed in a cold room with a temperature ranging from −10° C. to −17° C. At intervals of two weeks, one month, and two months, 5 per cent acidulated aqueous extracts were carefully made from the respective lots, sterilized, and assayed against the standard infundibular powder. At the end of the two weeks period the material kept at the higher temperature (−2° C. to +2° C.) assayed at nearly full theoretical value (6.3 mg. standard powder activity per cubic centimeter). The material at this time appeared good and presented no evidence of bacterial decomposition. At the end of one month it appeared grayish and showed distinct evidence of some putrefactive change. An extract made of the material at this time assayed at less than one-half the theoretical strength (3.3 mg. standard powder activity per cubic centimeter).

The infundibular lobes kept at the lower temperature (−10 C. to −17° C.) have shown no evidence of deviation from the normal in appearance, and extracts made therefrom at the stated intervals have assayed at full strength. An assay made of the 5 per cent extract of this material at the end of the two-months period showed an oxytocic activity equivalent to 7.0 mg. standard powder per cubic centimeter (see tracing 7).

CONCLUSIONS.

1. Infundibular extracts, carefully prepared and sterilized, do not depreciate in activity for at least one year, if kept in the cold room at a temperature of about 0° C.

2. Higher temperatures up to 37° C. (98° F.), and up to about two months do not affect the oxytocic or pressor activity of sterilized extracts, made either from fresh infundibular lobes or from desiccated standard material. Slight deterioration may occur after this period. The activity of the extract appears to withstand room temperature and diffuse sunlight for eight months without appreciable deterioration.

3. Slight deterioration in activity of the extract occurs at 45° C., and nearly complete destruction of its active principle or principles takes place after a period of three month's exposure at 60° C. As nearly as can be determined, the deterioration of pressor activity runs parallel to that of the oxytocic activity at the different periods

of exposure at this temperature. This observation lends support to the hypothesis of the chemical identity of the oxytocic and pressor principle.

4. Frozen fresh infundibular lobes retain their oxytocic activity for at least two months, if kept at a temperature of —10° to —17° C. At a temperature of about 0° C., the activity is not retained longer than about two weeks.

REFERENCES.

(1) Abel, J., and Nagayama, T.: J. Pharm. & Exper. Therap., v. 15, 1920, p. 347.
(2) Abel, J., and Rouiller, C. A.: Ibid. v. 20, 1922, p. 83.
(3) Smith, M. I., and McClosky, W. T.: Hyg. Lab. Bull. 138, p. 281.
(4) Tate, G.: Pharm. J., London, 1921, v. 106, p. 486.

HYGIENIC LABORATORY BULLETINS OF THE PUBLIC HEALTH SERVICE.

The Hygienic Laboratory was established in New York, at the Marine Hospital on Staten Island, August, 1887. It was transferred to Washington, with quarters in the Butler Building, June 11, 1891, and a new laboratory building, located in Washington, was authorized by act of Congress March 3, 1901.

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68TH CONGRESS
1st Session
HOUSE OF REPRESENTATIVES
DOCUMENT
No. 224

TREASURY DEPARTMENT
UNITED STATES PUBLIC HEALTH SERVICE

HYGIENIC LABORATORY—BULLETIN No. 139
NOVEMBER, 1924

I. THE USE OF COOKED MEAT MEDIUM FOR THE DETECTION OF C. TETANI

By IDA A. BENGTSON

II. STUDIES ON THE POTENCY TESTING OF PNEUMOCOCCUS VACCINES

By IDA A. BENGTSON

III. THE ADAPTABILITY OF VARIOUS AMERICAN PEPTONES FOR USE IN CHOLERA MEDIA

By IDA A. BENGTSON

WASHINGTON
GOVERNMENT PRINTING OFFICE
1924

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I. THE USE OF COOKED MEAT MEDIUM FOR THE DETECTION OF C. TETANI.[1]

By IDA A. BENGTSON, Associate Bacteriologist, Hygienic Laboratory, United States Public Health Service.

INTRODUCTION.

A study was undertaken to determine the value of cooked meat medium as compared with beef infusion broth in fermentation tubes for the detection of *C. tetani*.

Meat infusion broth in Smith fermentation tubes is the routine medium used for the testing of biologic products in the Hygienic Laboratory. The broth as used in fermentation tubes is adapted for the detection of organisms growing both aerobically and anaerobically. Just prior to use 1 cubic centimeter of a 1 per cent solution of glucose is added to each fermentation tube [2] which contains about 30 c. c. of broth, the tube then being heated in the Arnold sterilizer for a period of 30 minutes, and immediately tipped on removal from the sterilizer to expel the bubble of air. The closed arm affords suitable conditions for the growth of anaerobes, while aerobes may develop in the open arm.

The success attending the use of cooked meat medium in work undertaken by the writer in connection with the study of other anaerobes (*C. botulinum* and *C. parabotulinum*) suggested the possible superiority of the cooked meat medium over broth in fermentation tubes for the detection of *C. tetani*.

The cooked meat medium as used varies slightly from that described by Holman.[3] The history of the use of tissue in media for enhancing the growth of organisms from the time when first used by Theobald Smith (1890) has been reviewed by Holman.

The medium used by the writer is prepared as follows: Beef muscle is ground in a meat grinder and weighed. Two parts of water are added to one part of ground meat. The mixture is cooked in the Arnold sterilizer 1 hour, stirring occasionally. It is then removed from the sterilizer, filtered through filter paper, and allowed to drain for 1 hour. Both the meat residue and the filtrate are saved. The broth filtrate is adjusted to a reaction of pH 8.5. About 5 grams of meat are placed in each tube and 8–10 cubic centimeters of broth added. The tubes are then sterilized in the autoclave at 15 pounds

[1] Manuscript submitted for publication Feb. 25, 1924.

[2] This is for the purpose of enhancing the growth of *C. tetani*, the object being to add only a sufficient amount of glucose to provide a stimulus for growth, and at the same time not enough to bring about excessive acid production which is deleterious to the growth of *C. tetani* and the production of toxin.

[3] Jour. Bact., 1919, 4, 149–155.

pressure for 1½ hours. The following day the medium is heated in the Arnold sterilizer for a period of 1½ hours. Prior to this heating a cap of petrolatum (petroleum jelly, vaseline) about one-eight inch thick may have been added if desired. If the petrolatum cap is added at this time no subsequent heating is necessary. If the medium has been allowed to stand without the petrolatum cap before using, it is advisable to heat for one-half hour just prior to use in order to provide better anaerobic conditions. The reaction of the medium after sterilization and heating is about pH 6.8–7, which reaction seems to be retained even on further heating.

Infusion broth which has been found favorable for the growth of *C. tetani* is of a rich golden brown color. This apparently is a point of considerable importance, since light colored broths (pale straw color) have been found to be much less favorable.[4] The essential point seems to be that the meat infusion and subsequent processes should be carried out in such a way as to extract the greatest amount of substance from the meat. A reaction in the neighborhood of pH 7.6 has been used in the preparation of the various broths.

EXPERIMENTAL WORK.

Various methods of preparing the broths were first investigated.

The following table (Table I) shows the results obtained with broth in fermentation tubes prepared in two different ways. Media B_1 and B_3 were made by infusing the ground meat at refrigerator temperature for 24 hours without squeezing out the juice or prolonged draining; media B_2 and B_4 were prepared by cooking the meat instead of infusing, to extract the juice, the meat having been cut into pieces instead of being ground. Media B_1 and B_2 were made with Parke, Davis & Co.'s crystalline peptone and media B_3 and B_4 with Parke, Davis & Co.'s powdered peptone. Three different sets of tetanus spores were used—Nos. 28 and 29, which had been stored at a temperature of about 10° C. for a period of about 10 years, and No. 253, recently prepared spores suspended in salt solution.

TABLE I.—*Comparison of methods of preparing broth.*

[+=growth in closed arm; g=gas; b=small bubble of gas; superior figures indicate days on which growth and gas appeared.]

	Dilutions of spores.					
	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000	1/10,000,000
MEDIUM BROTH B_1.						
Spores 28	$+^{1}g^{2}$	$+^{1}g^{2}$	$+^{2}g^{2}$	$+^{2}g^{2}$	$+^{2}g^{2}$	----------
	$+^{1}$	$+^{1}g^{2}$	$+^{2}$	$+^{2}g^{2}$	$+^{2}g^{2}$	----------
Spores 29	$+^{2}g^{5}$	$+^{2}g^{2}$	$+^{2}g^{2}$	$+^{2}g^{4}$	----------	----------
	$+^{1}g^{4}$	$+^{2}g^{2}$	$+^{2}$	$+^{2}g^{2}$	----------	----------
Spores 253	$+^{1}b^{2}$	$+^{1}g^{2}$	$+^{1}g^{1}$	$+^{1}$	$+^{1}g^{2}$	$+^{1}$
	$+^{1}g^{1}$	$+^{1}g^{6}$	$+^{1}$	$+^{1}$	$+^{1}$	----------
	$+^{1}g^{2}$	$+^{1}g^{2}$	$+^{1}g^{2}$	$+^{1}g^{7}$	$+^{1}$	----------
	$+^{1}g^{2}$	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	----------

[4] An increase in the brownish color may, on the other hand, be due to overheating, and this should be avoided.

TABLE I.—*Comparison of methods of preparing broth*—Continued.

	Dilutions of spores.					
	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000	1/10,000,000
MEDIUM BROTH B_2.						
Spores 28	$+^1g^1$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$
	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$
Spores 29	$+^1g^1$	$+^2g^2$	$+^2g^2$			
	$+^2g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$		
Spores 253	$+^1$	$+^1b^2$	$+^1$	$+^1$	$+^1$	
	$+^1g^2$	$+^1g^2$	$+^1g^1$	$+^1$	$+^1$	
	$+^1g^1$	$+^1g^5$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$
	$+^1g^1$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$
MEDIUM BROTH B_3.						
Spores 28	$+^1$	$+^1g^2$	$+^2g^2$	$+^2$	$+^2g^6$	
	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$
Spores 29	$+^1$	$+^2g^2$	$+^2$	$+^2g^2$		
	$+^2g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$		
Spores 253	$+^1g^2$	$+^1g^2$	$+^1$	$+^1$	$+^2$	
	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^1g^2$
	$+^1g^2$	$+^1$	$+^1g^4$	$+^1$	$+^1$	$+^1g^2$
MEDIUM BROTH B_4.						
Spores 28	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$
	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$	
Spores 29	$+^1g^2$	$+^2g^2$	$+^2g^2$		$+^2g^2$	
	$+^2g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$		
Spores 253	$+^1g^2$	$+^1g^1$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$
	$+^1g^2$	$+^1g^1$	$+^1g^1$	$+^1$	$+^1g^2$	
	$+^1g^1$	$+^1g^2$	$+^1g^2$	$+^1g^4$	$+^1g^2$	
	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2$	

The results may be summarized thus:

	Number planted.	Number grew.	Per cent of growth.
Medium B_1 (meat infused without pressing out)	48	39	
Medium B_3 (meat infused without pressing out)	42	36	
	90	75	83.33
Medium B_2 (meat cooked in the liquid)	48	41	
Medium B_4 (meat cooked in the liquid)	48	40	
	96	81	84.38

The results show about equally good results with the two media prepared according to the two different methods.

Three other sets of broth were prepared, broth C being made with meat cut in pieces and cooked under 15 pounds pressure for 1 hour and allowed to stand over night before straining, broth D, with ground meat infused at ice-box temperature (8–10° C.) for 21 hours, strained and heated in streaming steam for 1 hour, and broth E made with ground meat heated to 70° C. for one-half hour in a double boiler and allowed to infuse for 20 hours at room temperature, then strained and heated in streaming steam for 1 hour. Spores 253 in dilutions at closer intervals than those used in the previous test were planted in the various broths and also in cooked meat medium. The following table (Table II) shows the results obtained:

TABLE II.—*Comparison of methods of preparing broth.*

Dilutions of spores.											
1/100,000	1/200,000	1/500,000	1/1,000,000	1/2,000,000	1/5,000,000	1/10,000,000	1/20,000,000	1/50,000,000	1/100,000,000	1/200,000,000	1/500,000,000
Broth C. (Cooked-meat broth, pH 7.5.)											
$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$					$+^2g^2$			
$+^1g^2$	$+^2g^2$	$+^2g^2$		$+^2g^3$			$+^2g^2$				
$+^1g^2$	$+^2g^2$	$+^2g^4$	$+^2g^2$								
$+^1g^2$	$+^1g^2$	$+^1g^3$	$+^2g^2$	$+^2g^2$	$+^3g^3$	$+^3g^3$					
$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$		$+^2g^2$					
Broth D. (Meat infusion; ice-box, pH 7.4.)											
$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$							
$+^2g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^1g^2$							
$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2$							
$+^1g^2$	$+^1g^2$	$+^1g^3$		$+^2g^2$							
$+^1g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$		$+^2g^2$	$+^2g^3$					
Broth E. (Meat infusion; heated to 70° C., then infused at room temperature, pH 7.4.)											
$+^1g^2$	$+^1g^3$	$+^2g^2$	$+^2g^3$								
$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$			$+^1g^2$				
$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^4$								
$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2$	$+^3g^3$	$+^3g^3$						
$+^2g^6$	$+^2g^2$	$+^2g^3$	$+^1g^2$	$+^1g^1$	$+^2g^3$						
Cooked meat medium No. 15.											
$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$				
$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$		$+^2g^2$			$+^2g^2$		
$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$						
$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$				
$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$		$+^2g^2$		$+^1g^2$		

The results may be summarized thus:

	Number of tubes planted.	Number of tubes which grew.	Per cent of growth.
Broth C	60	27	45.00
Broth D	60	25	41.66
Broth E	60	26	43.33
	180	78	43.33
Cooked meat medium	60	37	61.11

Here again there was very little difference in the results obtained in the three broths. The reaction of all was pH 7.4 or pH 7.5 and all were of a golden brown color. The results in the cooked meat medium were better than in any of the broths, as indicated by the figures 43.33 per cent of growth in the broth media and 61.11 per cent in the cooked meat medium. The highest dilution in which growth

occurred in the broth was 1:50,000,000, and in the cooked meat medium 1:100,000,000. The highest dilution in which growth occurred in all the tubes planted was 1:500,000 in the case of the broth and 1:2,000,000 in the case of the cooked meat medium.

The effect of standing after heating was tested on several different broths. It is to be expected that on long standing a certain amount of air will be absorbed by the medium and the conditions will be less favorable for the growth of anaerobic organisms. However, there was no marked difference in the growth obtained up to four hours. This is shown by the following test (Table III) which is one of several tests carried out:

TABLE III.—*Effect of length of time broth is left standing after heating.*

BROTH A_2.

Time of planting.	Dilutions of spores.					
	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000	1/10,000,000
12 m	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	----------
1 p. m	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^3$	----------
2 p. m	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$	----------	----------
3 p. m	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^1g^2$	----------	----------
4 p. m	$+^1g^2$	$+^2g^7$	$+^2g^2$	$+^2g^7$	$+^2g^3$	----------

That tetanus spores develop over a considerable range or reaction in broth which is in other respects a suitable one for the organisms is shown by the following test (Table IV). The reaction of the medium was originally pH 7.6. This was adjusted to the reactions indicated by the addition of N/1 NaOH or N/1 HCl.

TABLE IV.—*Effect of reaction.*

BROTH A_4.

pH.	Dilutions of spores.					
	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000	1/10,000,000
7.2	$+^1g^2$	$+^1g^1$	$+^1g^2$	$+^1$--	----------	$+^2g^2$
7.5	$+^1g^1$	$+^1g^1$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$
8.0	$+^1g^1$	$+^1g^1$	$+^1g^1$	$+^1g^2$	----------	$+^1g^2$
8.5	$+^1g^1$	$+^1g^1$	$+^1g^2$	$+^1g^2$	$+^1g^2$	----------
Unadjusted (pH 7.6)	$+^1g^1$	$+^1g^1$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$

A broth which, on the other hand, was not very favorable for the growth of the organisms due to improper methods of preparing the infusion (usually indicated by a pale color) was no more suitable at reactions which varied from the original reaction (pH 7.6) as widely as in the test shown above, than at the original reactions.

A test was put on to determine whether by the addition of one-tenth of 1 per cent of cystine, or a fragment of meat, to the broth, as

good results could be obtained as were obtained in the cooked meat medium. Broths B_1, B_2, B_3, and B_4 were used in the test. The results are shown in the following table (Table V):

TABLE V.—*Effect of adding cystine or glucose to broth.*

	Dilutions of spores.											
	1/1,000,000	1/2,000,000	1/5,000,000	1/10,000,000	1/20,000,000	1/50,000,000	1/100,000,000	1/200,000,000	1/500,000,000	1/1,000,000,000	1/2,000,000,000	1/5,000,000,000
0.1 PER CENT CYSTINE ADDED.												
Media B_1	$+^1$	$+^1$	$+^2$									
B_2	$+^1$	$+^1g^2$	$+^2$	$+^2$								
B_3	$+^2$	$+^1g^2$										
B_4	$+^1$	$+^1$	$+^2$	$+^2$								
FRAGMENT OF MEAT ADDED.												
Media B^1	$+^2g^3$	$+^1g^3$			$+^2g^3$			$+^2g^3$				
B^2	$+^2g^3$	$+^2g^2$		$+^2g^4$	$+^2$							
B^3	$+^2g^2$		$+^2$			$+^1$						
B^4	$+^2g^2$	$+^2g^2$					$+^1$			$+^2$		
NO MEAT OR CYSTINE ADDED.												
Media B^1		$+^1$										
B^2	$+^1$	$+^1g^2$			$+^1g^2$							
B^3												
B^4	$+^2$											
COOKED MEAT MEDIUM												
No. 5	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$						
	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$						
	$+^1g^1$	$+^1g^2$	$+^1g^2$	$+^1g^2$		$+^2g^2$						
No. 6	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$						
	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$		$+^1g^2$						
	$+^1g^1$	$+^1g^1$	$+^1g^2$	$+^1g^2$								
	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$					
	$+^1g^1$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$							
	$+^1g^1$	$+^1g^1$	$+^1g^2$	$+^1g^2$	$+^1g^2$							

The results obtained may be summarized thus:

Media.	Number of tubes planted.	Number of tubes which grew.	Per cent of growth.
Broth+0.1 per cent cystine	48	13	27.08
Broth+fragment of meat	48	15	31.25
Broth without addition of cystine or meat	48	5	10.42
Cooked meat	108	51	47.2

Though cystine or meat added to the broth made conditions more favorable for the growth of *C. tetani*, better growth was obtained in the cooked meat than in any of the broths.

The comparative tests of broth and the cooked meat medium as indicated in the preceding table and in Table II are examples of a number of similar tests, in all of which growth was obtained consistently in higher dilutions in the cooked meat medium than in the fermentation tubes containing broth.

In the preceding tests in which the cooked meat medium has been used the petrolatum cap was added before inoculation. Inoculation through the petrolatum cap is, however, inconvenient and requires considerable time in planting. A test was put on to determine the value of the petrolatum cap, with the following results:

TABLE VI.—*Value of petrolatum cap on cooked meat medium.*

Dilutions of spores.									
1/1,000,000	1/2,000,000	1/5,000,000	1/10,000,000	1/20,000,000	1/50,000,000	1/100,000,000	1/200,000,000	1/500,000,000	1/1,000,000,000
No petrolatum cap; spore suspensions planted by allowing them to drip from pipette without touching the medium.									
$+^{1}g^{2}$ $+^{1}g^{2}$	$+^{1}g^{2}$ $+^{1}g^{2}$	$+^{2}g^{3}$ $+^{1}g^{2}$	$+^{2}g^{2}$ $+^{2}g^{2}$	$+^{1}g^{2}$ ---	--- ---	$+^{3}g^{3}$ ---	--- ---	--- ---	--- ---
No petrolatum cap; spore suspension planted by lowering pipette into medium.									
$+^{1}g^{2}$ $+^{1}g^{2}$	$+^{2}g^{2}$ $+^{1}g^{2}$	$+^{1}g^{2}$ $+^{1}g^{2}$	$+^{2}g^{2}$ $+^{2}g^{2}$	--- $+^{2}g^{2}$	--- ---	$+^{2}g^{2}$ ---	--- ---	--- ---	--- ---
Petrolatum cap; spores suspensions planted by lowering pipette into medium.									
$+^{1}g^{2}$ $+^{1}g^{2}$	$+^{1}g^{2}$ $+^{1}g^{2}$	$+^{1}g^{2}$ $+^{1}g^{2}$	$+^{1}g^{2}$ $+^{1}g^{2}$	$+^{2}g^{2}$ $+^{1}g^{2}$	$+^{1}g^{2}$ ---	$+^{2}g^{2}$ $+^{2}g^{2}$	--- ---	--- ---	--- ---

The results may be summarized thus:

	Number of tubes planted.	Number of tubes which grew.	Per cent of growth.
No petrolatum cap (pipette not lowered into medium)	20	10	50
No petrolatum cap (pipette lowered into medium)	20	10	50
Petrolatum cap	20	13	65

Better results were therefore obtained with the vaseline cap than without.

A trial was made to determine whether as good results could be obtained by adding the petrolatum after planting as with medium to which a petrolatum cap had been added before inoculation. The following table (Table VII) shows the results:

TABLE VII.—*Effect of adding petrolatum to cooked meat medium before and after planting.*

	Dilutions of spores.											
	1/1,000,000	1/2,000,000	1/5,000,000	1/10,000,000	1/20,000,000	1/50,000,000	1/100,000,000	1/200,000,000	1/500,000,000	1/1,000,000,000	1/2,000,000,000	1/5,000,000,000
	PETROLATUM ADDED BEFORE PLANTING.											
No. 11	$+^2g^2$	$+^2g^2$	$+^2g^4$	$+^2g^2$	$^2g^4$							
	$+^2g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$								
No. 12	$+^2g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$		$+^4g^4$						
	$+^2g^2$	$+^2g^2$	$+^2g^4$	$+^2g^4$								
	$+^2g^2$	$+^2$	$+^2g^2$	$+^2g^2$	$+^2g^4$							
	$+^2g^4$	$+^2g^2$	$+^2g^2$	$+^2g^2$	$+^2g^4$	$+^2g^4$						
	$+^2g^2$	$+^2g^4$	$+^2g^2$	$+^2g^4$	$+^2g^2$							
	$+^2g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$								
	PETROLATUM ADDED AFTER PLANTING.											
No. 11	$+^2g^2$	$+^2g^2$	$+^4g^4$									
	$+^2g^2$	$+^2g^2$	$+^4g^4$	$+^2g^2$	$+^2g^4$		$+^2g^2$	$+^4g^4$				
No. 12	$+^2g^2$	$+^2g^2$	$+^2g^4$	$+^2g^2$		$+^2g^2$						
	$+^2g^2$	$+^2g^2$	$+^2g^4$	$+^2g^2$			$+^4g^4$					
	$+^2g^2$	$+^2g^2$	$+^2g^2$	$+^2g^5$								
	$+^2g^4$	$+^2g^4$	$+^4g^4$			$+^2g^2$						
	$+^2g^2$	$+^2g^2$		$+^2g^2$								
	$+^2g^2$	$+^2g^4$	$+^2g^2$	(a)								

[a] Tube broke.

The results may be summarized thus:

	Number of tubes inoculated.	Number of tubes which grew.	Per cent of growth.
Petrolatum added before planting	96	38	39.5
Petrolatum added after planting	96	34	35.41

The results are slightly in favor of adding the petrolatum before planting.

Another test was put on in which a comparison was made of the cooked meat medium without a petrolatum cap, of cooked meat medium to which the petrolatum cap was added after planting, and of three different lots of broth. The results are shown in the following table:

TABLE VIII.—*Comparison of cooked meat medium with and without petrolatum, and broth.*

Dilutions of spores.											
1/100,000	1/200,000	1/500,000	1/1,000,000	1/2,000,000	1/5,000,000	1/10,000,000	1/20,000,000	1/50,000,000	1/100,000,000	1/200,000,000	1/500,000,000
Cooked meat medium—no petrolatum added.											
$+^2g^2$ $+^1g^2$ $+^2g^2$	$+^2g^2$ $+^2g^2$ $+^2g^2$	$+^2g^2$ $+^2g^2$ $+^2g^2$	$+^2g^2$ $+^2g^2$ $+^2g^2$	$+^2g^2$ $+^2g^2$ ---	--- $+^2g^2$ $+^2g^2$	--- $+^2g^2$ ---	--- --- ---	$+^2g^2$ --- ---	--- --- ---	--- --- ---	--- --- ---
Cooked meat medium. Petrolatum added after planting.											
$+^1g^2$ $+^1g^2$ $+^1g^2$	$+^1g^2$ $+^2g^2$ $+^2g^2$	$+^2g^2$ $+^2g^2$ $+^2g^2$	$+^2g^3$ $+^3g^3$ $+^2g^2$	$+^2g^2$ $+^2g^2$ $+^3g^3$	$+^3g^3$ $+^3g^3$ $+^2g^2$	$+^3g^3$ $+^2g^2$ $+^3g^3$	--- $+^3g^3$ $+^3g^3$	--- --- ---	--- --- ---	--- --- ---	$+^3$ --- ---
Broth E.											
$+^1b^2$ $+^1$ $+^1g^2$	$+^1b^2$ $+^1$ $+^1$	$+^1$ --- $+^2$	--- $+^2$ ---	--- --- ---	--- --- ---	--- --- ---	--- $+^2g^2$ ---	--- --- ---	--- --- ---	--- --- ---	--- --- ---
Broth D.											
$+^1g^2$ $+^1g^2$ $+^1g^2$	$+^2$ $+^1g^2$ $+^1g^2$	$+^1g^2$ $+^1g^2$ ---	$+^1g^2$ $+^1b^2g^3$ $+^2g^2$	--- $+^1g^2$ ---	--- --- ---	--- $+^2g^2$ ---	--- $+^2g^2$ ---	--- --- ---	--- --- ---	--- --- ---	--- --- ---
Broth C.											
$+^1g^2$ $+^1b^2g^3$ $+^2$	$+^2g^2$ --- $+^2b^2g^3$	--- --- ---	$+^1g^2$ --- ---	--- --- ---	--- --- ---	--- --- ---	--- --- ---	--- --- ---	--- --- ---	--- --- ---	--- --- ---

The results may be summarized thus:

Media.	Number of tubes planted.	Number of tubes which grew.	Per cent of growth.
Cooked meat medium (no petrolatum added)	36	18	50.00
Cooked meat medium (petrolatum added after planting)	36	23	63.88
Broth E	36	10	27.77
Broth D	36	14	38.88
Broth C	36	6	16.66

The results with the cooked meat medium were considerably better than with the broth. The best results were obtained with the cooked meat medium to which petrolatum was added after planting. Though fairly good growth was obtained with the cooked meat media without petrolatum, in some of the tubes it was difficult to determine whether growth had taken place since the medium was not turbid. While theoretically the meat tubes without petrolatum would offer the best conditions for growth of both aerobes and anaerobes, the

medium would not be practical for the detection of *C. tetani* since in many cases it would be difficult to decide whether growth and gas had been produced or not.

The value of cooked meat medium for the detection of *C. tetani* was tested in two samples of vaccine virus 27 L and 29 H which had been rejected for human use.

Vaccine virus 29 H was inoculated in 0.01 cubic centimeter quantities in the cooked meat medium and three lots of broth. One set of tubes was inoculated with the uncontaminated virus and another with virus contaminated with dilutions of tetanus spores ranging from 1/100,000 to 1/500,000,000. The following table (Table IX) shows growth and gas production in the meat medium, and growth in the closed arm and gas production in the broth.

TABLE IX.—*Growth of C. tetani in vaccine virus.*

Dilutions of spores.											
1/100,000	1/200,000	1/500,000	1/1,000,000	1/2,000,000	1/5,000,000	1/10,000,000	1/20,000,000	1/50,000,000	1/100,000,000	1/200,000,000	1/500,000,000
COOKED MEAT MEDIUM PLANTED WITH VACCINE VIRUS CONTAMINATED WITH *C. TETANI*.											
$+^{1}g^{2}$ $+^{1}g^{2}$ $+^{1}g^{2}$	$+^{1}g^{2}$ $+^{1}g^{2}$ $+^{1}g^{2}$	$+^{1}g^{2}$ $+^{1}g^{2}$ $+^{3}g^{3}$	$+^{1}g^{2}$ $+^{1}g^{2}$ $+^{1}g^{2}$	$+^{1}g^{2}$ $+^{1}g^{2}$ $+^{1}g^{2}$	$+^{1}g^{2}$ $+^{1}g^{3}$ $+^{1}g^{4}$	$+^{1}g^{2}$ --- $+^{2}g^{2}$	$+^{2}g^{2}$ $+^{2}g^{2}$ $+^{2}g^{7}$	$+^{1}$ $+^{2}$ $+^{2}$	$+^{3}$ $+^{1}g^{1}$ ---	--- $+^{1}g^{2}$ $+^{1}$	$+^{1}$ --- $+^{1}g^{4}$
COOKED MEAT MEDIUM PLANTED WITH VACCINE VIRUS WITHOUT *C. TETANI* CONTAMINATION.											
$+^{2}$ $+^{2}$ $+^{2}$	$+^{2}$ $+^{1}$ ---	--- --- $+^{1}$	$+^{2}$ --- ---	--- --- ---	$+^{2}$ --- $+^{2}$	--- --- $+^{2}$	$+^{2}$ $+^{1}$ ---	$+^{1}$ --- $+^{1}$	--- --- ---	--- --- ---	$+^{1}$ $+^{1}$ ---
	Dilutions of spores.											
	1/100,000	1/200,000	1/500,000	1/1,000,000	1/2,000,000	1/5,000,000	1/10,000,000	1/20,000,000	1/50,000,000	1/100,000,000	1/200,000,000	1/500,000,000
BROTH PLANTED WITH VACCINE VIRUS CONTAMINATED WITH *C. TETANI*												
Broth E	$+^{1}g^{3}$	$+^{1}g^{3}$	$+^{1}$	$+^{2}g^{3}$	---	---	$+^{2}$	---	---	---	---	$+^{3}$
	$+^{1}g^{3}$	$+^{1}$	$+^{1}g^{2}$	$+^{1}g^{1}$	---	$+^{4}$	---	---	$+^{3}$	---	$+^{3}$	---
Broth D	$+^{1}b^{7}$	$+^{1}g^{2}$	$+^{1}g^{2}$	$+^{1}g^{3}$	---	---	$+^{1}g^{2}$	---	---	---	---	---
	$+^{1}g^{3}$	$+^{1}g^{3}$	$+^{3}g^{3}$	$+^{1}$	$+^{3}g^{3}$	---	---	---	---	---	$+^{1}g^{4}$	---
Broth C	$+^{1}g^{2}$	---	$+^{1}g^{2}$	---	---	---	---	---	---	$+^{3}$	---	---
	$+^{1}g^{2}$	$+^{1}g^{2}$	$+^{1}g^{2}$	---	$+^{1}g^{2}$	$+^{1}g^{2}$	---	---	---	---	---	---
BROTH PLANTED WITH VACCINE VIRUS WITHOUT *C. TETANI* CONTAMINATION.												
Broth E	---	---	---	---	---	$+^{7}$	---	---	$+^{2}$	---	---	---
	---	---	---	---	---	$+^{3}b^{7}$	---	---	---	---	$+^{2}$	---
Broth D	---	---	---	$+^{1}$	---	---	---	---	---	---	---	---
	---	---	---	---	---	---	---	---	---	---	---	---
Broth C	---	---	---	$+^{1}$	---	---	---	---	---	---	---	---
	---	---	---	---	---	---	---	---	---	---	---	---

Tests were made on mice with cultures which indicated the presence of *C. tetani* as evidenced by growth in the closed arm of the fermentation tube and by the production of gas in both media. The following table (Table X) shows the results of these tests (+ indicating tetanus in mice; – indicating *C. tetani* absent in tubes as shown by appearance of growth or negative results in mice):

TABLE X.—*Growth of C. tetani in vaccine virus.*

[Tests on mice.]

Dilutions of spores.											
1/100,000	1/200,000	1/500,000	1/1,000,000	1/2,000,000	1/5,000,000	1/10,000,000	1/20,000,000	1/50,000,000	1/100,000,000	1/200,000,000	1/500,000,000
COOKED MEAT MEDIUM PLANTED WITH VACCINE VIRUS CONTAMINATED WITH *C. TETANI.*											
+	+	+	+	+	+	+	+	–	–	–	–
+	+	+	+	+	+	–	–	–	–	+	–
+	+	+	+	+	+	+	–	–	–	–	–
COOKED MEAT MEDIUM PLANTED WITH VACCINE VIRUS WITHOUT *C. TETANI* CONTAMINATION.											
–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–
BROTH PLANTED WITH VACCINE VIRUS CONTAMINATED WITH *C. TETANI.*											
	Dilutions of spores.										
	1/100,000	1/200,000	1/500,000	1/1,000,000	1/2,000,000	1/5,000,000	1/10,000,000	1/20,000,000	1/50,000,000	1/100,000,000	1/200,000,000	1/500,000,000
Broth E	+	+	+	+	–	–	–	–	–	–	–	–
Broth D	+	+	+	+	–	–	+	–	–	–	–	–
Broth C	+	+	+	–	+	+	–	–	–	–	–	–
BROTH PLANTED WITH VACCINE VIRUS WITHOUT *C. TETANI* CONTAMINATION.												
Broth E	–	–	–	–	–	–	–	–	–	–	–	–
Broth D	–	–	–	–	–	–	–	–	–	–	–	–
Broth C	–	–	–	–	–	–	–	–	–	–	–	–

The results may be summarized thus:

Medium.	Number of tubes planted.	Number of tubes in which *C. tetani* grew.	Per cent of growth of *C. tetani*.
Cooked meat medium	36	23	63.88
Broth	72	25	34.72

The highest dilution in which *C. tetani* developed in the cooked meat medium was 1/200,000,000 and in the broth 1/10,000,000. The highest dilution in which *C. tetani* developed in all tubes planted was 1/5,000,000 in the case of the cooked-meat medium and 1/100,000 in the case of the broth.

The other sample of vaccine virus (27 L) contained an organism which produced a large amount of gas in the meat medium. Ninety-six tubes of the meat medium to which petrolatum was added before planting and 96 tubes of meat medium to which petrolatum was added after planting were inoculated with 0.1 cubic centimeter quantities of the vaccine virus contaminated with amounts of tetanus spores ranging from 1/100,000 cubic centimeter to 1/500,000,000 cubic centimeter. Twelve tubes of each set of meat tubes were also planted with 0.1 cubic centimeter quantities of uncontaminated vaccine virus. All tubes (100 per cent) showed growth and gas production after 48 hours incubation. Ninety-six tubes of broth without cystine and 96 tubes of broth containing 0.1 per cent cystine were also planted with spores as above. Fifty-nine of these showed 5 per cent or more of gas in the closed arm, 78 showed a bubble of gas after 3 days incubation. All except 3 showed growth in the closed arm. This vaccine virus was evidently quite heavily contaminated. In the cooked-meat medium it was not possible to distinguish by appearance of the growth or by smears as to the presence of *B. tetani*. No tests on mice were carried out with this set of tubes.

Another test was put on, using the cooked-meat medium, 0.01 cubic centimeter of the same vaccine virus being used for the inoculum. The dilutions of tetanus spores used for contaminating a portion of the vaccine virus ranged from 1/2,000,000 to 1/2,000,000,000. Ten tubes of the meat medium were planted each with 0.01 cubic centimeter of uncontaminated virus. All the tubes showed growth and gas production within 48 hours.

Tests carried out on mice gave the following results (Table XI), 0.1 cubic centimeter of the growth of each tube being inoculated subcutaneously (+ indicating the development of tetanus symptoms). It was necessary to test all the tubes since all showed gas.

TABLE XI.—*Growth of C. tetani in vaccine virus.*

Dilutions of spores.									
1/2,000,000	1/4,000,000	1/10,000,000	1/20,000,000	1/40,000,000	1/100,000,000	1/200,000,000	1/400,000,000	1/1,000,000,000	1/2,000,000,000
COOKED MEAT MEDIUM PLANTED WITH VACCINE VIRUS CONTAMINATED WITH *C. TETANI.*									
+	+	+	+	+	−	−	+	−	−
+	+	+	+	+	−	+	−	−	−
+	+	+	+	−	−	−	−	−	−
COOKED MEAT MEDIUM PLANTED WITH VACCINE VIRUS WITHOUT *C. TETANI* CONTAMINATION.									
−	−	−	−	−	−	−	−	−	−

The results may be summarized thus:

Medium.	Number of tubes planted.	Number of tubes in which *C. tetani* grew.	Per cent of growth of *C. tetani.*
Cooked meat	36	16	44.44

The results show that *C. tetani* grew and toxin was produced in dilutions as high or higher than in tests with pure cultures or with a vaccine virus containing few other organisms. The highest dilution in which growth occurred was 1/400,000,000 and the highest dilution in which growth occurred in all of the tubes inoculated was 1/20,000,000. The presence of other gas-producing organisms did not hinder the development of *C. tetani* or the production of toxin.

SUMMARY AND CONCLUSIONS.

The tables presented show that the cooked meat medium is more sensitive for the detection of *C. tetani* spores in pure culture than is broth in fermentation tubes.

In order to obtain the best results with the cooked meat medium in the detection of anaerobes it is advisable to use a petrolatum cap. This may be added before or after planting. An advantage of adding the petrolatum cap before planting is that further heating of the medium is obviated.

The results as applied to the sterility testing of biologic products indicate that the meat medium with a petrolatum cap is of distinct value in the detection of gas-producing anaerobes. When used with a petrolatum cap, however, conditions are not altogether favorable

for the growth of aerobic organisms, though it is probable that many of the latter are facultative anaerobes and would also develop in media suitable for anaerobes. The cooked meat medium without a petrolatum cap would offer conditions suitable for both aerobes and anaerobes but possibly somewhat at the expense of the anaerobes as compared with the previously capped medium. Gas production also can not be so readily observed since the gas is not confined, and this would probably necessitate the daily observation of the tubes. The growth does not always bring about a distinguishable turbidity and some tubes having growth might be overlooked.

The conclusion may therefore be drawn that the cooked meat medium with a petrolatum cap is useful as an adjunct to broth in fermentation tubes as a means for detecting gas-producing anaerobes in biologic products.

The results of the tests on two different samples of vaccine virus artificially contaminated with *C. tetani* indicate the superiority of the cooked meat medium over the broth for the detection of *C. tetani*, in this menstruum. In the case of one of the samples, few organisms were present, in the other gas-producing organisms were present. In the case of the former *C. tetani* was isolated in 63.88 per cent of cooked meat medium tubes and in 34.72 per cent of the broth tubes. In the latter *C. tetani* was isolated in 44.44 per cent of cooked meat medium tubes. (In this case higher dilutions of spores were used.) The results of both tests indicate that *C. tetani* can be detected in vaccine virus artificially contaminated with *C. tetani* spores in as high dilutions as with pure cultures. The presence of other gas-producing organisms did not hinder the development of *C. tetani* nor the production of toxin.

In addition to the fact that the cooked meat medium is more sensitive for the detection of *C. tetani*, another advantage lies in the fact that gas is always produced. This is not true of broth, a number of tubes planted with pure culture failing to show gas though growth occurred in the closed arm.

II. STUDIES ON THE POTENCY TESTING OF PNEUMOCOCCUS[1] VACCINES.

By IDA A. BENGTSON, Associate Bacteriologist, Hygienic Laboratory, United States Public Health Service.

INTRODUCTION.

The recent publications of Cecil and Steffen (1921, 1923) describing the successful vaccination of monkeys against pneumococcus pneumonia have served to arouse new interest in the subject of pneumococcus vaccine. The use of this vaccine for prophylaxis in man as practiced by Lister (1913, 1916, 1917) in South Africa and by Cecil and Austin, and Cecil and Vaughan in this country on recruits in the United States Army indicate that the product has a certain value. The results obtained were, however, not as conclusive as might be desired.

Further work by Cecil and his coworkers has served to elucidate some of the problems arising in connection with the use of pneumococcus vaccine. By means of intratracheal inoculations of pneumococcus cultures, Blake and Cecil found it possible to reproduce the clinical picture of human lobar pneumonia in monkeys and thus were able to establish a basis for determining experimentally the value of the vaccine in the prevention of the disease itself.

Cecil and Blake found in their early experiments that subcutaneous inoculations of monkeys with Type I pneumococcus vaccine in doses approximating those which had been employed in man did not afford protection against experimental pneumococcus Type I pneumonia. It was necessary to increase the dose considerably in order to produce immunity by the subcutaneous route as shown by Cecil and Steffen (1921). Intravenous inoculations with comparatively small doses were however successful.

The results obtained in man suggest that a higher degree of immunity would be secured with larger doses of vaccine. The severe reactions which sometimes occur in subcutaneous inoculation even with vaccines as dilute as those employed in the vaccination of soldiers in the United States Army by Cecil and Austin, and Cecil and Vaughan would militate against the use of very concentrated vaccines unless methods are devised for modifying the vaccine or the methods of administration.

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[1] Manuscript submitted for publication Feb. 25, 1924.

Cecil and Steffen (1922) report the successful immunization of monkeys against Type I pneumonoccus pneumonia by administering vaccine in large or small doses by the intratracheal route, and suggestive results were obtained by spraying the vaccine into the throat.

Perlzweig and Steffen report successful immunization of mice with modified pneumococcus vaccines consisting of the protein fraction obtained either by treating pneumococci with anhydrous sodium sulphate and dissolving the precipitate in dilute sodium carbonate solution or by dissolving pneumococci in bile salts, precipitating with alcohol and dissolving the precipitate in dilute alkaline solutions. Immunization could also be effected by the use of antigens isolated from each of the three fixed types of pneumococcus by tryptic digestion of the pneumococci and extraction of the digest with 70 to 90 per cent alcohol, the degree of immunity conferred being about equal to that obtained with the original saline vaccine.

The results reported above suggest that pneumococcus vaccine may become a prophylactic agent of practical value. This necessitates devising methods for testing the comparative value of such vaccines.

The work here reported was done following the publication of the early papers by Cecil and Austin (1918) and Cecil and Vaughan (1919). It was the purpose to determine the most suitable animal for testing purposes, using vaccines of known composition produced at the Hygienic Laboratory and then to test a number of commercial vaccines. In view of the fact that at the time this work was undertaken not enough information was at hand to decide what a standard vaccine should be, the work done should be regarded as an attempt to carry out certain tests for the purpose of throwing light on the general subject rather than as the definite working out of a specific method. As will be noted later, the commercial vaccines tested were of the greatest variety in their composition and in the dosage recommended.

For test animals rabbits and mice known to be susceptible to pneumococcus infection suggest themselves as being most useful for laboratory testing. As demonstrated by Cecil and Steffen (1921, 1923), monkeys are probably most suitable for determining the value of vaccine for human prophylaxis, but the use of monkeys for the routine testing of a considerable number of vaccines is not practicable.

If the rabbit could be used for the testing of pneumococcus vaccine in the same way as it is used for the testing of typhoid vaccine, i. e., by inoculating the animal and then testing the serum for agglutinins or other antibodies, the matter of testing would be comparatively simple. The use of three sets of animals would be avoided, as the same serum could be tested against all the three types of organisms. The simplicity of carrying out tests in this way, providing it could be shown that such tests were an index of the immunity

conferred, seemed to warrant the investigation of this method at considerable length. Most of the results obtained, however, showed that antibodies in the serum are present in relatively low concentration, and it is doubtful whether their demonstration would give a satisfactory test. In the following discussion some illustrations will be given showing the limitations of this method of testing.

The advantage of a test for active immunity as indicated by immunity against the live organism when inoculated into vaccinated animals is obvious except for the fact as just stated that large numbers of animals are required. Although the test is not the most rigid that might be used, i. e., protection against the disease as it occurs clinically, it is a test against pneumococcus infection, and it is assumed that this is an indication of its effectiveness against the disease itself.

The vaccines made by the Hygienic Laboratory were made with virulent cultures of the three different types of pneumococcus. The cultures were grown in glucose broth for 20–24 hours, after which the organisms were sedimented by centrifugalization. The organisms were suspended in a small amount of salt solution and heated to a temperature of 52–53° C. for one-half hour. The suspension was diluted to 2,500,000,000 or 3,000,000,000 organisms per cubic centimeter, using the Hopkins method of counting. Tricresol in a concentration of 0.25 per cent was used as preservative. In addition to the vaccines prepared thus, several commercial vaccines were used in the preliminary experimental work.

EXPERIMENTS WITH RABBITS.

Type I vaccine.—The tests made on rabbits include tests for active immunity as well as agglutination, precipitin and tropin tests on the serum, and bacteriostatic tests on the whole blood.

Active immunity.—The marked protection afforded in rabbits by three different Type I vaccines is shown in Table I. A series of six rabbits was inoculated intravenously with each of the three vaccines at intervals of 6 to 7 days, a dose of 1 cubic centimeter of the vaccine being injected each time. The various dilutions of live virulent culture were injected intraperitoneally 16 days after the last injection of vaccine.

TABLE I.—*Active immunity in rabbits inoculated intravenously with Type I pneumococcus vaccine.*

	Pneumococcus isolated.
VACCINE A.	
Rabbit 1. 0.00,000,1 cubic centimeter. Survived.	
2. 0.00,001 cubic centimeter. Survived.	
3. 0.00,01 cubic centimeter. Survived.	
4. 0.00,1 cubic centimeter. Survived.	
5. 0.01 cubic centimeter. Survived.	
6. 0.1 cubic centimeter. + 48 hr.	+

Pneumococcus isolated.

VACCINE B.

Rabbit 7. 0.00,000,1 cubic centimeter. Survived.
9. 0.00,001 cubic centimeter. Survived.
10. 0.00,01 cubic centimeter. Survived.
11. 0.00,1 cubic centimeter. Survived.
12. 0.01 cubic centimeter. Survived.

VACCINE C.

Rabbit 13. 0.00,000,1 cubic centimeter. Survived.
14. 0.00,001 cubic centimeter. Survived.
15. 0.00,01 cubic centimeter. Survived.
16. 0.00,1 cubic centimeter. Survived.
17. 0.01 cubic centimeter. Survived.
18. 0.1 cubic centimeter. Survived.

CONTROLS.

Rabbit 19. 0.00,000,1 cubic centimeter. +50 hr. +
20. 0.00,000,01 cubic centimeter +76 hr. +
21. 0.00,000,001 cubic centimeter. Survived.

The table shows that rabbits were protected against 100,000 fatal doses of Type I pneumococcus by vaccine A, against at least 100,000 fatal doses by vaccine B and against at least 1,000,000 fatal doses by vaccine C. The results are definite and pronounced, indicating a high degree of protection by the vaccines used against pneumococcus infection.

Agglutination tests on serums.—Seven days after the last injection of vaccine the rabbits were bled and agglutination tests carried out with the following results (Table II):

TABLE II.—*Agglutination tests on serums of rabbits inoculated intravenously with Type I pneumococcus vaccine (3 inoculations). (Tests made 7 days after last inoculation.)*

	Rabbit.	Dilutions of serum.					
		1/1	1/2	1/5	1/10	1/20	1/40
Series I—Vaccine A	1	[1] 2	2	2	2	1	0
	2	2	2	2	2	1	0
	3	2	2	2	2	0	0
	4	3	4	4	4	3	0
	5	3	4	4	4	3	1(?)
	6	1(?)	0	0	0	0	0
Series II—Vaccine B	7	2	2	1	1	0	0
	[2] 8						
	9	3	4	4	4	2	0
	10	3	3	4	3	1	0
	11	3	4	4	4	4	2
	12	1	1	1(?)	0	0	0
Series III—Vaccine C	13	2	3	3	3	1	0
	14	2	2	2	1	0	0
	15	3	3	3	1	0	0
	16	4	4	4	3	3	1
	17	2	2	3	4	3	2
	18	2	2	2	1	0	0
Normal rabbit		0	1	0	0	0	0
Antigen without serum, 0.							

[1] 4 indicates complete agglutination; 3 about 75% agglutination; 2 about 50% agglutination, and 1 about 25% agglutination.
[2] Rabbit died.

About two months after the date of the last inoculation seven rabbits were bled and agglutination tests again carried out with antigen A (vaccine A), with the results shown in Table III. The rabbits had meanwhile received varying small doses of living pneumococcus culture as shown in Table I.

TABLE III.—*Agglutination tests on serums of rabbits inoculated intravenously with Type I pneumococcus vaccine (3 inoculations). (Tests made 2 months after last inoculation of vaccine.)*

	Dilutions of serum.					
	1/1	1/2	1/5	1/10	1/20	1/40
Rabbit 1	0	0	0	0	0	0
Rabbit 4	4	3	2	0	0	0
Rabbit 7	1(?)	0	0	0	0	0
Rabbit 11	4	3	2	1	0	0
Rabbit 12	0	0	0	0	0	0
Rabbit 17	4	4	3	1	0	0
Rabbit 18	0	0	0	0	0	0
Normal rabbit	0	1	1	0	0	0

Antigen without serum, 0.

The results obtained in the two tests indicate a rather low agglutinin response. A decrease in the agglutinin titer is shown by the second test. The results in the two tests are consistent in that in general the rabbits which showed the best agglutinin response in the first test continued to do so also in the second test.

The results on the whole however are not very definite. Agglutination was present in only low dilutions and there was considerable variation among the different rabbits. The indications are that the agglutinin response is not sufficient to be demonstrated satisfactorily when three injections of vaccines with the doses employed are made. It is possible with a larger number of injections somewhat higher agglutination titers could be shown.

Bacteriostatic tests.—The use of these tests was suggested by the work of Heist, Solis-Cohen and Solis-Cohen; and Heist and Solis-Cohen. As pointed out by Bull and Bartual the action of whole blood on pneumococci is bacteriostatic rather than bactericidal. As stated by these authors, "cultures of fresh whole blood of immune animals, as compared with cultures in the blood of susceptible animals, show a greatly prolonged latent period, and, in a general way, the relative lengths of the latent periods of the cultures correspond to the relative resistances of the animals to infection by these organisms."

Tests were carried out according to the method of Heist, Solis-Cohen and Solis-Cohen. The test as performed by these authors is essentially as follows: Graded dilutions of an 18-hour broth culture are drawn, up to a fixed point, into sets of five or more capillary pipettes of about 1 millimeter diameter, the culture being immediately withdrawn by touching the end of the pipette to a piece of sterile gauze. A film of culture is thus left on the inner wall of the tube.

After drying for a few minutes in the air, the tube is filled to the fixed point with blood from the rabbit, the end of the pipette being touched to the drop of blood as it flows from the puncture in the ear vein. The capillary tubes are then sealed, incubated and smears made from the contents. The presence of pneumococci in the smear indicates multiplication of the organism. The absence of the organism indicates bactericidal or bacteriostatic action of the blood. As explained by Bull and Bartual, the inhibition of the multiplication of the organisms is dependent on opsonization of the pneumococci by the immune serum and phagocytosis of the organisms by the polynuclear cells. In any case it is necessary to fix certain standards of amounts of culture used, time of incubation, etc. in order to obtain comparable results.

In the tests carried out by the writer, the method of bleeding the rabbit in the afternoon and making smears the following morning was adopted. The following table (Table IV) illustrates the results obtained with Type I pneumococcus in blood from rabbit 17, which had withstood the inoculation of 0.01 cubic centimeter of live culture, and from rabbit 18, which had withstood the inoculation of 0.1 cubic centimeter culture. (See Table I.)

TABLE IV.—*Bacteriostatic tests on blood of rabbits inoculated intravenously with Type I pneumococcus vaccine (3 inoculations).*

[+ indicates pneumococci in smears; − indicates pneumococci not in smears.]

Rabbit.	Dilutions of culture.					
	0.1	0.01	0.00,1	0.00,01	0.00,001	0.00,000,1
No. 17	+	+	−	−	−	−
No. 18	+	+	−	+	−	−
Normal No. 1	+	[1] +	+	+	−	−
Normal No. 2	+	+	+	[1] +	−	−

[1] Few.

The culture used was a virulent one, killing mice on a dose of 0.00,000,001 cubic centimeter. The differences between the vaccinated and normal rabbits are not very marked, though there is a suggestion of a difference between them.

Similar tests were carried out on two United States Army vaccines and several commercial vaccines, tests being made against all three types of organism. The results with two such vaccines are shown in the accompanying tables. (Tables V and VI.) In certain cases the number of injections of vaccine was increased to six. A modification of the technique of Heist, Solis-Cohen and Solis-Cohen was used in several of the tests. This consisted of the use of larger amounts of culture and blood, agglutination tubes being used in place of capillary tubes. A small amount of culture dilution was placed in the tube and a given number of drops of blood were collected into the tube from the ear vein of a rabbit. This method did not, however, afford more satisfactory results than the first method used.

TABLE V.—*Bacteriostatic tests on blood of rabbits inoculated intravenously or subcutaneously with polyvalent pneumococcus vaccine (3 inoculations).*

[Inoculations of vaccine Oct. 25, Nov. 1, Nov. 8. Test Nov. 18.]

	Dilutions of culture.				
	0.1	0.01	0.001	0.00,01	0.00,001
INTRAVENOUS INOCULATION OF VACCINE.					
Rabbit 1a:					
Type I	−	−	−	−	−
II	−	−	−	−	−
III	+	+	−	−	−
Rabbit 2a:					
Type I	+	+	−	−	−
II	+	−	−	−	−
III	+	−	−	−	−
SUBCUTANEOUS INOCULATION OF VACCINE.					
Rabbit 19a:					
Type I	−	−	−	−	−
II	−	−	−	−	−
III	−	−	−	−	−
Rabbit 23a:					
Type I	−	−	−	−	−
II	−	−	−	−	−
III	+	−	−	−	−
Control rabbit 1a:					
Type I	+	+	+	+	−
II	+	+	−	+	+
III	+	+	+	+	+
Control rabbit 2a:					
Type I	+	+	+	+	+
II	+	+	+	−	−
III	+	+	+	−	−

TABLE VI.—*Bacteriostatic tests on blood of rabbits inoculated intravenously with a polyvalent commercial vaccine.*

[Inoculations of vaccine Nov. 11, 19, 25, Dec. 2. Test Dec. 11–12.]

	Dilutions of culture.					
	0.1	0.01	0.001	0.0001	0.00,001	0.000,000,1
VACCINATED.						
Rabbit 1b:						
Type I	+	−	−	−	−	−
II	+	+	+	+	−	−
III	+	+	+	+	Few	−
Rabbit 2b:						
Type I	+	+	−	−	−	−
II	+	+	+	+	+	−
III	+	+	+	+	Few	−
Rabbit 6b:						
Type I	+	+	+	Few	+	+
II	+	+	+	+	+	Few
III	+	+	+	+	Few	Few
Rabbit 11b:						
Type I	+	+	Few	Few	−	−
II	+	+	+	Few	+	−
III	+	+	+	+	Few	−
Rabbit 17b:						
Type I	+	+	−	Few	−	−
II	+	+	+	Few	−	−
III	+	+	Few	Few	−	−
Rabbit 4b:						
Type I	+	Few	−	−	−	−
II	Few	+	Few	+	Few	−
III	+	+	+	−	−	−
Rabbit 5b:						
Type I	+	Few	−	−	−	−
II	−	Few	+	−	+	−
III	−	+	Few	−	Few	−
Control rabbits:						
No. 1b	+	+	+	Few	−	−
	+	+	+	+	Few	Few
	+	+	+	+	Few	+
No. 2b	+	+	+	Few	−	−
	+	+	+	+	+	−
	+	+	+	+	Few	−
No. 3b:	+	+	+	+	+	Few
	+	+	+	+	+	+
	+	+	+	+	Few	Few

Regarding the practical value of the test, it may be stated that while the results are suggestive and perhaps in some cases rather definite, still it does not compare in definiteness with the direct protection test.

Precipitin and tropin [2] *tests.*—These tests were carried out on the serums of sets of rabbits inoculated with several different vaccines. In a set of 8 rabbits inoculated intravenously 4 times with a polyvalent commercial vaccine the tropin test was negative on 2 rabbits, and positive on 6 and among these 1 was strongly positive. The precipitin test was found to be positive occasionally in low dilutions of serum but no definite results were obtained. Of these two tests it may be said that in certain cases the results were suggestive and that it might be possible to distinguish between serums from vaccinated and from control rabbits but that for ascertaining the comparative value of different vaccines these two methods were of little value.

Summarizing the results obtained by the various methods used in testing the Type I vaccines, it is obvious that the test for active immunity is by far the most pronounced. The results were so definite that it would decidedly be the method of choice for testing Type I vaccines. A series of 6 rabbits with 2 or 3 control rabbits for determining the virulence of the culture would furnish a satisfactory test for such vaccines.

Type II and III vaccines.—The satisfactory results obtained in the testing of Type I vaccines by the active immunity test on rabbits could not be duplicated with Type II and III vaccines. Although it was possible to show protection against infection with these types in a number of cases, the results were often unsatisfactory for the reason that the virulence of these cultures for rabbits fluctuated very greatly or was very low. The cultures of all three types were almost invariably fatal to mice in dilutions of 0.00,000,01 and 0.00,000,001 cubic centimeters. The same amount of Type I culture which was fatal to rabbits was fatal to mice. The following table (Table VII) shows the results of tests on the virulence of the Type I culture for rabbits over a period of six months.

TABLE VII.—*Virulence of Type I pneumococcus for rabbits.*

Date.	Dose.	Results.
	Cubic centimeters.	
Feb. 24, 1919	0. 00, 000, 001	+42 hours.
	0. 00, 000, 001	+42 hours.
Mar. 19, 1919	0. 00, 000, 01	+22 hours.
July 9, 1919	0. 00, 000, 01	+17 hours.
	0. 00, 000, 001	+31 hours.
Aug. 21, 1919	0. 00, 000, 01	+42 hours.
	0. 00, 000, 001	+24 hours.
Aug. 23, 1919	0. 00, 000, 01	+37 hours.
	0. 00, 000, 001	+38½ hours.
Aug. 27, 1919	0. 00, 000, 1	+50 hours.
	0. 00, 000, 01	+76 hours.
	0. 00, 000, 001	Survived.

[2] Tropin tests carried out by Miss Alice C. Evans.

Types II and III, on the other hand, were much less virulent for rabbits. A number of attempts were made to determine the virulence of these cultures for rabbits but the results were not satisfactory. In the case of Type II irregularities were usually present. An illustration of this is shown in the following table:

TABLE VIII.—*Virulence of Type II pneumococcus for rabbits and mice.*

Rabbit.	Weight.	Dose.	Results.	Organism.
	Grams.	*Cubic centimeters.*		
1c	3,000	0.1	Survived	
2c	2,500	0.01	+2 days	Pneumococcus isolated.
3c	2,500	0.00,1	+4 days	Pneumococcus not isolated.
4c	2,150	0.00,01	Survived	
5c	1,950	0.00,00,1	+2 days	Pneumococcus isolated.
6c	1,930	0.00,000,1	+2 days	Pneumococcus isolated.
7c	1,850	0.00,000,01	+1 day	Pneumococcus not isolated.
8c		0.00,000,001	Survived	

Mouse.	Dose.	Results.	Organism isolated.
	Cubic centimeter		
1	0.00,01	Chloroformed after 6 hours	Pneumococcus.
2	0.00,001	+23 hours	Pneumococcus.
3	0.00,000,1	+23 hours	Pneumococcus.
4	0.00,000,01	+27 hours	Pneumococcus.
5	0.00,000,001	Survived	

Attempts to stabilize the virulence of the culture by growing in rabbit blood and by passage through rabbits failed.

Type III was only slightly virulent for rabbits. The following results (Table IX) were obtained with the same culture of Type III injected into mice and rabbits.

TABLE IX.—*Virulence of Type III pneumococcus for rabbits and mice.*

	Dose.	Rabbits.	Mice.	Organism isolated.
	Cubic centimeters.			
9c	0.1	+90 hours		Pneumococcus.
10c	0.01	Survived		
11c	0.00,1	Survived		
12c	0.00,01	Survived	+42 hours	Pneumococcus.
13c	0.00,001	Survived	+42 hours	Pneumococcus.
14c	0.00,000,1	Survived	+42 hours	Pneumococcus.
15c	0.00,000,01	Survived	Survived	
16c	0.00,000,001	Survived	Survived	

The amounts of culture fatal to the rabbits also fluctuated somewhat, varying from 0.1 cubic centimeter to 1 cubic centimenter in different tests.

The irregular results shown above indicate the difficulty of obtaining any satisfactory results with cultures which are so variable in virulence. The accompanying protocol (Table X) shows the results with Types II and III Hygienic Laboratory vaccines, the rabbits having been inoculated three times intravenously with vaccine containing 3,000,000,000 organisms per cubic centimeter and the cultures inoculated 13 days after the last inoculation of vaccine.

TABLE X.—*Active immunity in rabbits inoculated intravenously with Type II and III vaccines.*

TYPE II.

	Weight.	Amount of culture.	Result.	Pneumococcus isolated.
Vaccinated rabbits:	*Grams.*	*Cubic centimeters.*		
No. 6d	1,660	0.1	Survived	
No. 5d	1,700	0.01	Survived	
No. 3d	1,840	0.00,1	+45 hours	(+)
No. 2d	1,910	0.00,01	Survived	
No. 4d	2,280	0.00,001	Survived	
No. 1d	2,410	0.00,000,1	Survived	
Control rabbits (no vaccine):				
No. 14d	1,660	0.1	+93 hours	(+)
No. 16d	1,720	0.01	+12 days	(−)
No. 18d	1,920	0.001	Survived	
No. 17d	2,100	0.00,01	Survived	
No. 21d	2,120	0.00,001	Survived	
No. 19d	2,430	0.00,000,1	Survived	

TYPE III.

Vaccinated rabbits:				
No. 12d	1,700	1	+7 days	(−)
No. 7d	1,750	0.5	Survived	
No. 10d	1,825	0.1	Survived	
No. 8d	1,900	0.01	Survived	
No. 9d	1,945	0.001	+76 hours	(+)
No. 11d	1,945	0.00,01	Survived	
Control rabbits (no vaccine):				
No. 22d	1,700	1	Survived	
No. 20d	1,850	0.5	+24 hours	(+)
No. 13d	2,070	0.1	+5 days	(−)
No. 23d	2,100	0.01	Survived	
No. 24d	2,180	0.00,1	Survived	
No. 15d	2,500	0.00,01	Survived	

EXPERIMENTS WITH MICE.

Type I vaccine.—Tests were carried out on mice with the same experimental vaccines which were used on rabbits and which had afforded very high protection. Several series of tests were made, using mice which had been vaccinated with the vaccine 1, 2, and 3 times, the intervals between vaccinations being 4 to 6 days and the culture being given about the same length of time after the last inoculation of vaccine. The results are shown in the accompanying table (Table XI):

TABLE XI.—*Active immunity tests on mice inoculated intraperitoneally with Type I pneumococcus vaccines.*

Amount of culture.	One inoculation.		
	Vaccine A.	Vaccine B.	Controls.
0.00,01 cubic centimeter	+27 hours	+27 hours	+27 hours.
0.00,001 cubic centimeter.	Survived	+2 hours	+19 hours.
0.00,000,1 cubic centimeter.	Survived	Survived	+45 hours.
0.00,000,1 cubic centimeter.	Survived	Survived	+43 hours.
0.00,000,01 cubic centimeter.	+2 hours	Survived	+29 hours.
0.00,000,01 cubic centimeter.	Survived	Survived	+31 hours.
0.00,000,001 cubic centimeter	Survived	Survived	+45 hours.
0.00,000,001 cubic centimeter	Survived	Survived	+43 hours.
	Protection against 1,000 fatal doses.	Probable protection against 1,000 fatal doses.	

TABLE XI.—*Active immunity tests on mice inoculated intraperitoneally with Type I pneumococcus vaccines*—Continued.

Amount of culture.	Two inoculations.		
	Vaccine A.	Vaccine B.	Controls.
0.01 cubic centimeter.	+17 hours	+27 hours	
0.01 cubic centimeter.	Survived	+66 hours	
0.00,1 cubic centimeter.	+27 hours	Survived	
0.00,1 cubic centimeter.	+27 hours	+24 hours	
0.00,01 cubic centimeter.	Survived	Survived	
0.00,01 cubic centimeter.	Survived	Survived	
0.00,001 cubic centimeter.	Survived	Survived	
0.00,001 cubic centimeter.	Survived	Survived	
0.00,000,01 cubic centimeter.			+42 hours.
0.00,000,01 cubic centimeter.			+42 hours
0.00,000,001 cubic centimeter.			+48 hours.
0.00,000,001 cubic centimeter.			+48 hours.
	Protection against 10,000 fatal doses.	Protection against 10,000 fatal doses.	

Amount of culture.	Three inoculations.		
	Vaccine A.	Vaccine B.	Controls.
0.1 cubic centimeter.	+19 hours	+19 hours	
0.1 cubic centimeter.		+21 hours	
0.01 cubic centimeter.	Survived	+22 hours	
0.01 cubic centimeter.		+21 hours	
0.00,1 cubic centimeter.	Survived	+21 hours	
0.00,1 cubic centimeter.		+74 hours	
0.00,01 cubic centimeter.	Survived	Survived	
0.00,01 cubic centimeter.		Survived	
0.00,000,1 cubic centimeter.			+28 hours.
0.00,000,1 cubic centimeter.			+35 hours.
0.00,000,01 cubic centimeter.			+43 hours.
0.00,000,01 cubic centimeter.			+43 hours.
0.00,000,01 cubic centimeter.			Survived.
0.00,000,001 cubic centimeter.			Survived.
0.00,000,001 cubic centimeter.	Protection against 1,000,000 fatal doses.	Protection against 1,000 fatal doses.	

The following summarizes the results:

	One inoculation.	Two inoculations.	Three inoculations.
	Fatal doses.	*Fatal doses.*	*Fatal doses.*
Protection afforded by vaccine A	1,000	10,000	1,000,000
Protection afforded by vaccine B	1,000	10,000	1,000

Tests on mice—Types II and III vaccines.—Several series of mice were inoculated with Type II and III vaccines, inoculations being made 4 or 5 days apart and the culture inoculated about 5 days after the last inoculation of vaccine. The following results (Table XII) were obtained with a Type II vaccine:

TABLE XII.—*Active immunity tests on mice inoculated intraperitoneally with Type II pneumococcus vaccine (2 inoculations).*

Amount of culture.	Vaccinated mice.	Controls.
0.1 cubic centimeter	+20 hours	
0.1 cubic centimeter	+20 hours	
0.01 cubic centimeter	+24 hours	
0.01 cubic centimeter	+24 hours	
0.00,1 cubic centimeter	+(44 hours	
0.00,1 cubic centimeter	+(44 hours	
0.00,01 cubic centimeter	+(44 hours	
0.00,01 cubic centimeter	+(44 hours	
0.00,001 cubic centimeter	+(44 hours	+(44 hours.
0.00,001 cubic centimeter	+(44 hours	+(44 hours.
0.00,000,1 cubic centimeter	+(44 hours	+(44 hours.
0.00,000,1 cubic centimeter	Survived	+(44 hours.
0.00,000,01 cubic centimeter	Survived	+(44 hours.
0.00,000,01 cubic centimeter	Survived	+(44 hours.
0.00,000,001 cubic centimeter	Survived	Survived.
0.00,000,001 cubic centimeter	Survived	Survived.

Protection was afforded against 1 to 10 fatal doses.

A test of another Type II vaccine gave the following results: (Table XIII) the vaccine being injected twice with an interval of 3 days between the injections. The culture was administered 4 days after the last injection of vaccine.

TABLE XIII.—*Active Immunity tests on mice inoculated intraperitoneally with Type II pneumococcus vaccine (2 inoculations).*

Amount of culture.	Vaccinated mice.	Controls.
0.01 cubic centimeter	+32 hours	
0.01 cubic centimeter	+47 hours	
0.00,1 cubic centimeter	+32 hours	
0.00,1 cubic centimeter	+35 hours	
0.00,01 cubic centimeter	+35 hours	
0.00,01 cubic centimeter	+33 hours	
0.00,001 cubic centimeter	+(46 hours	
0.00,001 cubic centimeter	+(46 hours	
0.00,000,1 cubic centimeter	+(46 hours	+32 hours.
0.00,000,1 cubic centimeter	+(46 hours	
0.00,000,01 cubic centimeter	Survived	+35 hours.
0.00,000,01 cubic centimeter	Survived	
0.00,000,001 cubic centimeter	+53 hours	+35 hours.
0.00,000,001 cubic centimeter	+37 hours	
0.00,000,000,1 cubic centimeter	Survived	+37 hours.
0.00,000,000,1 cubic centimeter	Survived	

Some protection was shown in that a control mouse died on the dose of 0.00,000,000,1 cubic centimeter, while the vaccinated mice on 0.00,000,01 cubic centimeter survived.

A test with a Type III vaccine (3 billion organisms per cubic centimeter) showed the following results (Table XIV). The mice were given two inoculations 4 days apart and the culture administered 4 days after the last inoculation.

TABLE XIV.—*Active immunity tests on mice inoculated intraperitoneally with Type III pneumococcus vaccine (2 inoculations).*

Amount of culture.	Vaccinated mice.	Controls.
0.01 cubic centimeter	+35 hours	
0.00,1 cubic centimeter	+29 hours	
0.00,01 cubic centimeter	+27 hours	
0.00,001 cubic centimeter	+27 hours	
0.00,000,1 cubic centimeter	Survived	+37 hours.
0.00,000,01 cubic centimeter	+66 hours	+41 hours.
0.00,000,001 cubic centimeter	+41 hours	+40 hours.
0.00,000,000,1 cubic centimeter	Survived	Survived.

The test shows some protection in that the vaccinated mouse receiving 0.00,000,1 cubic centimeter survived, although the mice on the next two smaller doses died.

Another Type III vaccine also containing 3,000,000,000 organisms per cubic centimeter was tested with the following results (Table XV):

TABLE XV.—*Active immunity tests on mice inoculated intraperitoneally with Type III pneumococcus vaccine (2 inoculations).*

Amount of culture.	Vaccinated mice.	Controls.
0.1 cubic centimeter	+20 hours	
0.1 cubic centimeter	+20 hours	
0.01 cubic centimeter	+26 hours	
0.01 cubic centimeter	+44 hours	
0.00,1 cubic centimeter	+26 hours	
0.00,1 cubic centimeter	+44 hours	
0.00,01 cubic centimeter	+44 hours	
0.00,01 cubic centimeter	+44 hours	
0.00,001 cubic centimeter	+44 hours	+44 hours.
0.00,001 cubic centimeter	+44 hours	+44 hours.
0.00,000,1 cubic centimeter	+44 hours	+44 hours.
0.00,000,1 cubic centimeter	+44 hours	+44 hours.
0.00,000,01 cubic centimeter	+44 hours	+44 hours.
0.00,000,01 cubic centimeter	Survived	Survived.
0.00,000,001 cubic centimeter	Survived	Survived.
0.00,000,001 cubic centimeter	Survived	Survived.
0.00,000,000,1 cubic centimeter	Survived	Survived.
0.00,000,000,1 cubic centimeter	Survived	Survived.

In this test apparently no protection was afforded with the Type III vaccine.

The experimental work with Types II and III vaccines on mice indicate that much less protection was afforded by the particular vaccines used than was afforded by the Type I vaccine. The results with Type II are probably better than those with Type III.

The injections of these two vaccines in the doses used (1,500,000,000 organisms at each injection) seemed to act adversely on the mice, as indicated by the roughened fur and appearance of unthrift. That better results could be obtained with a much more dilute vaccine is suggested by the following test (Table XVI). The mice received one inoculation of vaccine and 10 days later cultures were injected. One series of mice received a vaccine containing 100,000,000 organisms per cubic centimeter and the other contained 500,000,000.

TABLE XVI.—*Active immunity tests on mice inoculated intraperitoneally with Type II vaccines diluted.*

VACCINE A (100,000,000 ORGANISMS).

Amount of culture.	Vaccinated mice.	Controls.
0.00,1 cubic centimeter	Survived	0.00,000,1; +22 hours.
0.00,1 cubic centimeter	Survived	0.00,000,1; +36 hours.
0.00,01 cubic centimeter	Survived	0.00,000,01; +46 hours.
0.00,01 cubic centimeter	Survived	0.00,000,01; survived.
0.00,001 cubic centimeter	Survived	0.00,000,001; survived.
0.00,001 cubic centimeter	Survived	0.00,000,001; survived.
0.00,000,1 cubic centimeter	+46 hours (pneumococcus)	
0.00,000,1 cubic centimeter	Survived	
0.00,000,01 cubic centimeter	Survived	
0.00,000,01 cubic centimeter	Survived	

VACCINE B (500,000,000 ORGANISMS).

0.00,1 cubic centimeter	Survived
0.00,1 cubic centimeter	+31 hours (pneumococcus)
0.00,01 cubic centimeter	+22 hours (pneumococcus)
0.00,01 cubic centimeter	+46 hours (pneumococcus)
0.00,001 cubic centimeter	Survived
0.00,001 cubic centimeter	+30 (not pneumococcus)
0.00,000,1 cubic centimeter	+46 (pneumococcus)
0.00,000,1 cubic centimeter	+48 (pneumococcus)
0.00,000,01 cubic centimeter	+46 (pneumococcus)
0.00,000,01 cubic centimeter	+46 (pneumococcus)
0.00,000,001 cubic centimeter	Survived
0.00,000,001 cubic centimeter	Survived

In certain of the tests made on the polyvalent commercial vaccines much higher protection was afforded against Types II and III, particularly Type II, than was found in the tests made with the experimental vaccines injected separately and the results did not seem to bear any definite relation to the concentration of the vaccine. It is possible that there is a certain amount of cross protection, i. e., high protection in the case of Type I may afford some protection against Types II and III.

In accordance with the results of the experimental work carried out it seemed advisable to use mice in making tests on the commercial vaccines. The virulence of the three types can be maintained at fairly definite levels. A larger number of animals can also be employed than if rabbits were used.

In order to limit the tests to a reasonable number of mice it seemed best to arrange the tests somewhat as follows:

Twenty-four [3] vaccinated and twelve control mice to be used in testing a vaccine which contains the three types of pneumococcus. This allows for eight dilutions of each culture for the vaccinated mice, ranging from 0.01 cubic centimeter to 0.00,000,000,1 cubic centimeter and for four dilutions of each culture for the control mice ranging from 0.00,000,1 cubic centimeter to 0.00,000,000,1 cubic centimeter. Two inoculations to be given 4–5 days apart, and the culture dilutions the same length of time after the last inoculation of vaccine.

[3] As a rule 10 or 12 extra mice were vaccinated to allow for deaths before the cultures were inoculated and for repetitions of doubtful tests.

The following protocols (Table XVII–XVIII) illustrate tests put on in this way, the first protocol showing a test on a vaccine which afforded protection against all three types, and the second a test on one which afforded no protection whatever. In stating the amount of protection afforded by a given vaccine it seems necessary to consider survivals which occur in irregular order as well as those occurring in regular order. In the case of the control unvaccinated mice it was found as a rule that the deaths occur in regular order (not necessarily as to number of hours however), i. e., if a mouse died on a certain dilution of the culture all mice on lower dilutions also died. In the case of the vaccinated mice, however, mice on certain of the lower dilutions sometimes survived, while those on the smaller doses succumbed. This occurred so frequently that it seemed to be of some significance. Accordingly these survivals were treated as if they had occurred in order.

The point may be illustrated under Type III in Table XVII. The mouse receiving 0.00,000,01 cubic centimeter which survived is considered in place of the mouse receiving 0.00,000,001 cubic centimeter which died, i. e., the two are reversed in order and the statement may be made that protection was afforded against one fatal dose.

TABLE XVII.—*Test of pneumococcus vaccine.*

[Inoculation of mice: Jan. 7, 0.5 cubic centimeter vaccine intraperitoneally; Jan. 11, 0.5 cubic centimeter vaccine intraperitoneally; Jan. 15, 10 a. m., cultures.]

TYPE I.

Amount of culture.	Vaccinated mice.	Control mice.	Protection against—
0.01 cubic centimeter	+46 hours		
0.00,1 cubic centimeter	Survived		
0.00,01 cubic centimeter	Survived		
0.00,001 cubic centimeter	Survived		
0.00,000,1 cubic centimeter	Survived	+43 hours	1,000,000 fatal doses of Type I.
0.00,000,01 cubic centimeter	+47 hours [a]	+51 hours	
0.00,000,001 cubic centimeter	Survived	+76 hours	
0.00,000,000,1 cubic centimeter	Survived	+37 hours	

TYPE II.

0.01 cubic centimeter	+67 hours		
0.00,1 cubic centimeter	+39 hours		
0.00,01 cubic centimeter	+41 hours		
0.00,001 cubic centimeter	Survived		
0.00,000,1 cubic centimeter	+47 hours	+51 hours	1 fatal dose of Type II.
0.00,000,01 cubic centimeter	+59 hours	+31 hours	
0.00,000,001 cubic centimeter	+71 hours	+56 hours	
0.00,000,000,1 cubic centimeter	Survived	Survived	

TYPE III.

0.01 cubic centimeter	+56 hours		
0.00,1 cubic centimeter	+43 hours		
0.00,01 cubic centimeter	+41 hours		
0.00,001 cubic centimeter	+43 hours		
0.00,000,1 cubic centimeter	+46 hours	+27 hours	
0.00,000,01 cubic centimeter	Survived	+51 hours	1 fatal dose of Type III.
0.00,000,001 cubic centimeter	+56 hours	+51 hours	
0.00,000,000,1 cubic centimeter	Survived	Survived	

[a] Pneumococcus not isolated.

TABLE XVIII.—*Test of pneumococcus vaccine.*

[Inoculations of mice: Oct. 23, 1920, 0.5 cubic centimeter intraperitoneally; Oct. 26, 1920, 0.5 cubic centimeter intraperitoneally; Oct. 30, 1920, 0.5 cubic centimeter cultures.]

TYPE I.

Amount of culture.	Vaccinated mice.	Control mice.	Protection against—
0.01 cubic centimeter	+46 hours		
0.00,1 cubic centimeter	+42 hours		
0.00,01 cubic centimeter	+42 hours		
0.00,001 cubic centimeter	+48 hours		
0.00,000,1 cubic centimeter	+38 hours		
0.00,000,01 cubic centimeter	+48 hours	+34 hours	
0.00,000,001 cubic centimeter	+38 hours	+48 hours	1 fatal dose.
0.00,000,000,1 cubic centimeter	Survived	+60 hours	

TYPE II.

0.01 cubic centimeter	+26 hours		
0.00,1 cubic centimeter	+26 hours		
0.00,01 cubic centimeter	+26 hours		
0.00,001 cubic centimeter	+42 hours		
0.00,000,1 cubic centimeter	+26 hours		
0.00,000,01 cubic centimeter	+34 hours	+26 hours	
0.00,000,001 cubic centimeter	Survived	Survived	0 fatal dose.
0.00,000,000,1 cubic centimeter	Survived	Survived	

TYPE III.

0.01 cubic centimeter	+38 hours		
0.00,1 cubic centimeter	+50 hours		
0.00,01 cubic centimeter	+46 hours		
0.00,001 cubic centimeter	+46 hours		
0.00,000,1 cubic centimeter	+34 hours		
0.00,000,01 cubic centimeter	+48 hours	+42 hours	
0.00,000,001 cubic centimeter	Survived	Survived	0 fatal dose.
0.00,000,000,1 cubic centimeter	Survived	Survived	

The results obtained in testing other commercial vaccines according to this method are shown in tabular form at the end of the article.

DISCUSSION.

The results of the tests carried out on the commercial vaccines as well as the experimental work on the vaccines of known composition show that the amount of protection afforded to mice may be measured rather definitely and comparison made as to the relative value of the vaccines in question. It would be reasonable to assume that a vaccine which gave entirely negative results in the test used would not be of value as a prophylactic agent. The indication would be that there was something faulty in the method of preparation of the vaccine. If protection were afforded against 100,000 to 1,000,000 or even a smaller number of fatal doses of culture the indications would be that such a vaccine would be of some definite value.

In general the vaccines tested produced considerable immunity against Type I pneumococcus (with the exception of several which showed no protection whatever against any of the types). As in the case of pneumococcus serum, so in pneumococcus vaccine most satisfactory results are to be expected with Type I. Only slight

protection was afforded by the vaccines against Type III pneumococcus. Type II appeared to occupy an intermediate position in relation to the other two.

Regarding the amounts of vaccine suitable for testing on mice it was thought that the most information could be obtained by using in the preliminary work uniform amounts of vaccine as to number of organisms and using the same amounts as to volume in testing the commercial vaccines. In the preliminary experimental work vaccines containing 3 billion organisms per cubic centimeter were used for the most part. The mice uniformly received 0.5 cubic centimeter of vaccine intraperitoneally at each inoculation and the rabbits either 0.5 or 1 cubic centimeter for the first and 1 cubic centimeter for the second and third inoculations. A series of tests was carried out on mice in which the Hygienic Laboratory vaccines were diluted, the mice being inoculated with vaccines containing 50,000,000, 100,000,000, 250,000,000, 500,000,000, 1,000,000,000, and 1,500,000,000 organisms. A commercial vaccine containing 10,000,000,000 organisms was diluted to 2,500,000,000 and 500,000,000 organisms, and tests carried out with the original and with the diluted vaccines. The commercial vaccines tested ranged from 100,000,000 to 20,000,000,000 organisms per cubic centimeter. On comparing the results obtained in all of these tests it appears that no definite statements can be made as to what concentration of organisms is most favorable for affording protection in mice. It seems that a vaccine which is decidedly dilute may afford very good protection. Good results were also obtained with very concentrated vaccines though at times it appeared that the doses used may have been overwhelming and what may be likened to a negative phase brought out. This seemed to be particularly true of Types II and III vaccines.

The matter of testing pneumococcus vaccines for their probable value for man would seem therefore to be a question of determining either on man or monkeys the concentration and dose of vaccine which is most likely to be of value and then so adapting the test to mice as best to show this value. This would probably involve diluting the vaccine to a certain point before injecting into mice. A dose which is suitable for man seems quite likely to be an overwhelming one for a mouse.

As to the length of immunity conferred as the result of the use of pneumococcus vaccine some information was obtained incidental to this work as far as animals are concerned. Tests were carried out at intervals on the surviving rabbits in the series of 18 rabbits referred to in the first table and also on another series of rabbits. It was found that six months after the administration of the vaccine protection was afforded against 100 fatal doses, as against 100,000 fatal doses 16 days after the last inoculation of vaccine. After 9 months there was practically no protection.

SPECIFICITY.

The possible cross protection between the different types has been referred to. A test was also carried out with a staphylococcus vaccine to determine the specificity of pneumococcus vaccines as regards other organisms. A series of mice was inoculated with this vaccine in the same way as with the pneumococcus vaccine. No protection whatever was afforded against Types I, II, or III pneumococcus cultures.

In conclusion it may be stated, considering the results as a whole, that apparently the potency testing of pneumococcus vaccine can be carried out with reasonably satisfactory results. Though irregularities are to be expected, the experiments reported show some decidedly definite and clear-cut results. The test for active immunity is undoubtedly the most effective test and the superiority of mice over rabbits has been shown.

It is evident that the protective property afforded as the result of vaccination resides in the serum only to a limited extent and although it can not be demonstrated very satisfactorily through the presence of agglutinins, tropins, precipitins, or bacteriostatic properties, it may nevertheless exist and can be shown by inoculating the vaccinated animal itself with the live culture. This test is decisive, while in carrying out tests for determining the presence of the antibodies mentioned the impression is gained that although these tests may give some information, they are too uncertain to be of great practical value. The same may be said in general of tests for determining passive immunity by protection tests on other animals than those receiving the vaccine. In work previously reported by the writer in testing the comparative value of saline and oil vaccines the mouse protection test was made use of to some extent and the results while giving some information were not very striking. Mouse protection tests with serum from inoculated persons were also used by Cecil and his coworkers in their early work and a certain number of positive results were obtained. The recent publications of Cecil and associates show, however, that in monkeys there may be complete immunity against pneumonia, though protective antibodies are very often absent in the serum.

SUMMARY.

Tests were carried out on rabbits and mice to determine their suitability for testing the potency of pneumococcus vaccine.

Agglutination, precipitin and tropin tests were made on the serum and tests designed to show bacteriostatic action were made on the whole blood of vaccinated rabbits. Tests for determining active immunity in rabbits using Type I pneumococcus vaccine were also carried out. The tests for active immunity as shown by direct

inoculation of the live cultures in the vaccinated animals were by far the most conclusive. Tests on the serum and whole blood were suggestive but not sufficiently definite for practical use.

Tests for active immunity in rabbits produced by Types II and III vaccine were inconclusive owing to the fluctuating or low virulence of these types for rabbits.

Tests for active immunity produced in mice were found to be most satisfactory for the testing of polyvalent pneumococcus vaccines containing Types I, II, and III. The superiority of mice over rabbits was due to the fact that the virulence of the three fixed types can be maintained at a very uniform level for this species.

In testing Types I, II, and III experimental vaccines separately in mice, the best results were obtained with Type I vaccine. Only a small amount of protection was afforded against Type III. Decidedly less protection was afforded by Type II than by Type I vaccine. In the testing of polyvalent vaccines, more definite protection was afforded against Types II and III, suggesting the possibility of a certain amount of cross protection.

Immunity in rabbits against Type I pneumococcus lasted to a moderate extent for 6 months and disappeared in 9 months.

Commercial polyvalent pneumococcus vaccines afforded protection in mice against doses of culture ranging from 0 to 1,000,000 fatal doses in the case of Type I, 0 to 10,000 fatal doses in the case of Type II and 0 to 100 fatal doses in the case of Type III.

Tests on commercial pneumococcus vaccines.

Vaccine.	Dose.	Number of inoculations.	Total dosage.	Dates of inoculations of vaccine.	Date of inoculation of culture.	Protection afforded (fatal doses).		
						Type I.	Type II.	Type III.
	Cubic centimeter.			1919.	1919.			
A	0.5	2	1 cubic centimeter (750,000,000 organisms).	Nov. 14, 21	Nov. 29	1,000	10	10
				1920.	1920.			
B	0.5	2	1 cubic centimeter (3,000,000,000 organisms).	Mar. 19, 23	Mar. 27	1,000	10	1
C	0.5	2	1 cubic centimeter (400,000,000 organisms.)	June 3, 7	June 12	1	10,000	0
D	0.5	2	1 cubic centimeter	July 9, 13	July 17	1,000,000	100	10
E	0.5	2	1 cubic centimeter (10,000,000,000 organisms).	July 22, 26	July 31	1,000,000	1–10	0
F	0.5	2	1 cubic centimeter (1,000,000,000 organisms).	Aug. 10, 14	Aug. 18	100	10,000	10
G	0.5	2	1 cubic centimeter (20,000,000,000 organisms).	Oct. 23, 26	Oct. 30	1	0	0
H	0.5	2	1 cubic centimeter (3,000,000,000 organisms).	Nov. 11, 15	Nov. 20	10–100	1,000	1
I	0.5	2	1 cubic centimeter (9,000,000,000 organisms).	Nov. 11, 15	Nov. 20	100	1–10	0

Tests on commercial pneumococcus vaccines—Continued.

Vaccine.	Dose.	Number of inoculations.	Total dosage.	Dates of inoculations of vaccine.	Date of inoculation of culture.	Protection afforded (fatal doses).		
						Type I.	Type II.	Type III.
	Cubic centimeter.			1920.	1920.			
K	0.5	2	1 cubic centimeter	Dec. 3, 7	Dec. 12	100	0	10
L	0.5	2	1 cubic centimeter (4,000,000,000 organisms).	Dec. 3, 7	Dec. 12	1,000	1,000	1,000
M	0.5	2	1 cubic centimeter (20,000,000,000 organisms).	Dec. 14, 22	Dec. 28	100	100	1
N	0.5	2	1 cubic centimeter (500,000,000 organisms).	Dec. 14, 24	Dec. 28	1,000	1,000	0
O	0.5	2	1 cubic centimeter (1,000,000,000 organisms).	Dec. 30 1921. Jan. 4	1921. Jan. 8	0	0	0
P	0.5	2	1 cubic centimeter (100,000,000 organisms).	1920. Dec. 31 1921. Jan. 4	Jan. 8	10	100	1
Q	0.5	2	1 cubic centimeter	Jan. 7, 11	Jan. 15	1,000,000	1	1
R	0.5	2	1 cubic centimeter (500,000,000 organism).	Jan. 7, 11	Jan. 15	10,000	10,000	1
S	0.5	2	1 cubic centimeter (1,000,000,000 organisms)	Feb. 11, 15	Feb. 10	0	0	0
T	0.5	2	1 cubic centimeter (1,000,000,000 organisms).	Feb. 18, 24	Mar. 1	0	0	0
U	0.5	2	1 cubic centimeter (3,000,000,000 organisms).	Mar. 5, 9	Mar. 12	10	100	100
V	0.5	2	1 cubic centimeter (4,000,000,000 organisms).	Mar. 5, 9	Mar. 12	1,000	10,000	100

BIBLIOGRAPHY.

Bengtson, Ida A. 1920. Potency of bacterial vaccines suspended in oil. Hyg. Lab. Bull., No. 122, pp. 33–34.

Blake, F. G., and Cecil, R. L. 1920. Studies in experimental pneumonia. I. Production of pneumococcus lobar pneumonia in monkeys. Jour. Exper. Med., v. 31, pp. 403–443.

Bull, C. G., and Bartual, L. 1920. Pneumococcus cultures in whole fresh blood I. The retardative effect of the blood of immune animals and the mechanism of the phenomenon. Jour. Exper. Med., v. 31, pp. 233–251.

Cecil, R. L., and Austin, J. H. 1918. Results of prophylactic inoculation against pneumococcus in 12,519 men. Jour. Exper. Med., v. 28, pp. 19–41.

Cecil, R. L., and Blake, F. G. 1920. Studies on experimental pneumonia. IV. Results of prophylactic vaccination against pneumococcus pneumonia in monkeys. Jour. Exper. Med., v. 31, pp. 519–553.

Cecil, R. L., and Steffen, G. I. 1921. Studies on pneumococcus immunity. I. Active immunization of monkeys against pneumococcus Type I pneumonia with pneumococcus Type I vaccine. Jour. Exper. Med., v. 34, pp. 245–258.

Cecil, R. L., and Steffen, G. I. 1922. Vaccination of monkeys against pneumococcus Type I pneumonia by means of intratracheal injections of pneumococcus Type I vaccine. Pub. Health Rep., v. 37, pp. 2735–2744.

——— 1923. Studies on pneumococcus immunity. II. Active immunization of monkeys against pneumococcus, Types II, III, and IV pneumonia with the homologous pneumococcus vaccine. Jour. Exper. Med., v. 38, pp. 149–161.

Cecil, R. L., and Vaughan, H. T. 1919. Results of prophylactic vaccination against pneumonia at Camp Wheeler. Jour. Exper. Med., v. 29, pp. 457–483.

Heist, G. D., Solis-Cohen, S., and Solis-Cohen, M. 1918. The bactericidal action of whole blood with a new technique for its determination. Jour. Immunol., v. 3, pp. 261–276.

Heist, G. D., and Solis-Cohen, S. 1919. The bactericidal action of the whole blood of rabbits following inoculations of pneumococcus bacterins. Jour. Immunol., v. 4, pp. 147–166.

Hopkins, J. G. 1913. A method of standardizing bacterial vaccines. Jour. Amer. Med. Assoc., v. 60, pp. 1615–1617.

Lister, F. S. 1913. Specific serological reactions with pneumococci from different sources. (So. African Inst. for Med. Res, No. 2).

——— 1916. An experimental study of prophylactic inoculation against pneumococcal infection in the rabbit and in man. (So. African Inst., for Med. Res., No. 8).

——— 1917. Prophylactic inoculation of man against pneumococcal infections and more particularly against lobar pneumonia. (So. African Inst. for Med. Res., No. 10.)

Perlzweig, W. A., and Steffen, G. I. 1923. Studies on pneumococcus immunity. III. The nature of pneumococcus antigen. Jour. Exper. Med., v. 38, pp. 163–182.

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III. THE ADAPTABILITY OF VARIOUS AMERICAN PEPTONES FOR USE IN CHOLERA MEDIA.[1]

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INTRODUCTION.

The work here reported was undertaken for the purpose of ascertaining whether the various American peptones can be used as effectively as Witte's peptone, called for in Goldberger's [2] formulæ for cholera media. The opportunity was also afforded in this connection to study in terms of H ion concentration the reaction most favorable for the development of *V. choleræ*.

The advantage of the Goldberger media lies in the simplicity and ease of their preparation and the availability of the ingredients. The particular advantage lies in the fact that Goldberger found it unnecessary to adjust the reaction except empirically. This may be explained by the following considerations: Approximate uniformity in reaction of Witte's peptone; the use of beef extract (Liebig's) and not meat infusion in the media; the fact that the cholera vibrio develops over a considerable range of reaction and that alkaline egg mixtures inhibit *B. coli*.

Dunham's solution unadjusted in reaction is usually recommended as a favorable enrichment medium to be used prior to streaking on plates for isolation. Many cholera cultures probably have a natural tendency to overgrow *B. coli* in Dunham's solution, particularly if they are numerous. Here the question of reaction probably does not enter in greatly, unless the peptone is one which produces a rather acid medium. If, on the other hand, the strain is one which does not have a tendency to multiply rapidly or is present in small numbers, it is necessary to make conditions more favorable for the growth of the cholera organism by rendering them less favorable for *B. coli*. Advantage is taken of the fact that *V. choleræ* is able to develop at a reaction which is more alkaline than is tolerated by *B. coli*, and it is in this connection that the alkaline egg enrichment medium of Goldberger is of particular value.

This paper is mainly concerned with the two enrichment media, which Goldberger recommends be used conjointly, and the alkaline egg agar plating medium adapted from a similar medium described earlier by Krumwiede, Prátt and Grund.[3]

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[1] Manuscript submitted for publication Oct. 4, 1923.

[2] Hygienic Laboratory Bulletin, 1914, No. 91, pp. 19–39.

[3] Jour. Infect. Dis., 1912, 10, 134–141.

EXPERIMENTAL WORK.

The peptone enrichment solution of Goldberger, which is modified Dunham's solution, is prepared as follows :

Potassium nitrate	1 gram.
Sodium carbonate (crystallized)	2 grams.
Sodium chloride	100 grams.
Peptone (Witte)	100 grams.
Distilled H_2O	1,000 cubic centimeters.

Dissolve by heating, then filter and distribute in flasks in 100 cubic centimeter quantities; sterilize and store. For use this stock solution is diluted by the addition of 9 volumes of distilled water and this solution distributed in tubes and flasks in quantities of 10 and 50 cubic centimeters, respectively, and sterilized.

The alkaline egg peptone enrichment solution is made as follows:

(*a*) Prepare an alkaline egg solution by first shaking up or beating up an egg with an equal volume of water and then adding to this egg water an equal volume of a 5 per cent solution of anhydrous sodium carbonate. Steam ¾ to 1 hour. (*b*) Prepare Dunham's solution: Peptone 10 grams, salt 5 grams, water 1,000 cubic centimeters. For use mix (*a*) and (*b*) in proportion of 1 to 9.

The alkaline egg agar medium for plating is prepared as follows:

1. Mix thoroughly equal parts of whole egg and water. Filter through thin layer of cotton.

2. Add to No. 1 an equal volume of 6.5 per cent solution of sodium carbonate (anhydrous) and mix thoroughly. Steam in Arnold sterilizer 20 minutes.

3. Prepare an agar as follows: Meat extract (Liebig's) 3, peptone (Witte) 10, sodium chloride (chemically pure) 5, glucose 1, agar 30, distilled (or good quality tap) water 1,000.

Mix 1 part of No. 2 with 5 of boiling hot No. 3 and pour the plates. Allow plates to dry with covers off.

The peptone enrichment solution made according to the above formula contains only 0.0074 per cent of Na_2CO_3 and can not be considered a very alkaline medium. The egg enrichment medium contains 0.25 per cent of Na_2CO_3 and the alkaline egg agar 0.54 per cent. The alkaline egg agar medium of Krumwiede, Pratt, and Grund contains from 0.67 to 0.75 per cent Na_2CO_3, which, however, was found by Goldberger to be too alkaline for the growth of the cholera strains which he studied.

In this paper the formulæ of Goldberger were followed and variations in reactions effected by the use of added normal sodium hydroxide or normal hydrochloric acid.

The strains used include three laboratory strains, No. 87 which is the Naples strain 159, used by Goldberger; No. 95, which is the Austria 13 strain, received from the New York City health department; and No. 93, which was received from the American Museum of Natural History and previously obtained by them from the New York City health department. Two other strains used by Goldberger, No. 88 (Naples 202 strain) and No. 97 (New York 1132)

were also used in part of the work. Later 3 strains recently isolated from cases of cholera in the Philippine Islands and 6 strains recently isolated in the cholera epidemic in Poland were added. In addition to these 1 culture of *B. coli*, Hygienic Laboratory strain 119, 2 cultures of *B. coli* recently isolated from stools, 1 culture of *B. alkaligenes*, Hygienic Laboratory strain 116, and a culture of *B. pyocyaneus*, Hygienic Laboratory strain 279, were used.

The peptones used included the five American peptones Parke, Davis & Co.'s, Fairchild's, Armour's, Difco, and Squibb's, with Witte's as control. As a rule the American peptones produce more acid media than Witte's. This was found to be true of all except Squibb's.[4]

Though there are other factors to be considered the main part of the work resolved itself into ascertaining to what extent differences in reaction of the various peptones used affect the results and to what extent it is necessary or desirable to modify the reactions of the various media. A special study was made of the growth of *V. choleræ* and *B. coli* in the alkaline range above pH 8.

The general plan of the work may be summarized in the following paragraphs:

1. A study was made of the growth curves of *V. choleræ*, *B. coli*, *B. pyocyaneus*, and *B. alkaligenes*, after cultivation in Witte's peptone alkaline egg enrichment medium adjusted to varying reactions.

2. Having determined the favorable reaction for the growth of *V. choleræ* and the suppression of *B. coli* in Witte's alkaline egg peptone medium, tests were carried out to find whether it was desirable to adjust media made with the various American peptones to a corresponding reaction.

3. Tests similar to those described in the preceding paragraph, were made with mixtures of *V. choleræ* and *B. coli*.

4. Determination of the reaction of Witte's alkaline egg agar plating medium suitable for the growth of *V. choleræ* and inhibitory to *B. coli*.

5. Growth of *V. choleræ* and *B. coli* on alkaline egg agar plates, using the various peptones in two series, the agar being unadjusted in one series, and in the other adjusted to the reaction which had been found favorable in the tests carried out with Witte's peptone.

6. Isolation of the cholera organism. Having obtained information from the preceding tests as to conditions favorable for the growth of *V. choleræ*, the methods were put into practice for isolating the organism from artificially contaminated stools.

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[4] The following peptones were used: Witte's pre-war peptone, not numbered; Squibb's 1E06234; Fairchild's, 170620; Difco, not numbered; Armour's, 9-8-17; Parke, Davis & Co.'s, 231795 and 2486609. (The results for cultures 400 and 404 in Table II were obtained with Parke, Davis & Co.'s peptone No. 2522835.)

1. Growth curves of *V. choleræ*, *B. coli*, *B. alkaligenes*, and *B. pyocyaneus* in Witte's alkaline egg enrichment medium:

The alkaline egg enrichment medium was selected as a medium for determining the growth curves of the various organisms for the reason that it was found to be fairly stable in pH value in the alkaline range. Peptone solutions adjusted to reactions more alkaline than pH 8 were found to be very unstable, the reaction always tending in the direction of neutrality, the more so as the degree of alkalinity was increased. This was observable on sterilization, on standing at room or incubator temperature, and on inoculation with cultures.

It was felt after some work had been done on the subject that the determination of suitable buffer agents to be added to the peptone solution to overcome this difficulty was scarcely justifiable in view of the fact that the alkaline egg medium apparently provided the buffer components necessary to maintain the desired reaction within reasonable limits without the addition of other substances. Though not as stable as might be desired in the more acid reactions used the changes were much less in proportion than in the peptone solutions. Reactions between pH 8.5 and 10 were found to be fairly stable.

The pH value of alkaline egg enrichment medium made with Witte's peptone was about pH 9.5–9.6. Different portions of this medium were adjusted to reactions of pH 8.0, 8.5, 9.0, and 10 by the addition of N/NaOH and N/HCl. The following table and diagrams indicate the results obtained on planting with 0.001 cubic centimeter of the various cultures, incubating for seven hours and plating on glucose agar. (Table I; Diagrams I–II.)

TABLE I.—*Growth of V. choleræ and B. coli in Witte's alkaline egg peptone enrichment media.*

Original pH.	*V. choleræ* 87.			*V. choleræ* 88.			*V. choleræ* 97.		
	Number of organisms per cubic centimeter.	Log.	pH.	Number of organisms per cubic centimeter.	Log.	pH.	Number of organisms per cubic centimeter.	Log.	pH.
8	15,600,000	7.19	8.6	16,800,000	7.23	8.6	8,850,000	6.95	8.6
8.5	980,000	5.99	8.8	2,700,000	6.43	8.8	465,000	5.67	8.6
9	1,710,000	6.23	9.0	1,000,000	6.0	9.0	1,820,000	6.26	8.8
9.5	112,000	5.05	9.4	263,000	5.42	9.2	285,000	5.45	9.2
10	0	------	9.7	0	------	9.6	84,000	4.92	9.7

Original pH.	*V. choleræ* 93.			*V. choleræ* 95.			*B. coli* 119.		
	Number of organisms per cubic centimeter.	Log.	pH.	Number of organisms per cubic centimeter.	Log.	pH.	Number of organisms per cubic centimeter.	Log.	pH.
8	100	2	8.6	2,630,000	6.42	8.0	9,250	3.97	8.6
8.5	23,500	4.37	8.6	16,900,000	7.23	8.0	5,100	3.71	8.6
9	42,000	4.62	9.4	38,800,000	7.59	8.4	1,200	3.08	9.3
9.5	91,500	4.96	9.4	14,100,000	7.15	9.2	200	2.3	9.4
10	300	2.48	10.0	445,000	5.65	10	0	------	9.7

TABLE I.—*Growth of V. choleræ and B. coli in Witte's alkaline egg peptone enrichment media*—Continued.

RECENTLY ISOLATED STRAINS.

Original pH.	*V. choleræ* 409.			*V. choleræ* 400.			*V. choleræ* 399.		
	Number of organisms per cubic centimeter.	Log.	pH.	Number of organisms per cubic centimeter.	Log.	pH.	Number of organisms per cubic centimeter.	Log.	pH.
8	6,250,000	6.8	8.6	4,670,000	6.67	8.6	54,000,000	7.73	8.0
8.5	2,820,000	6.45	8.9	15,400,000	7.19	8.7	31,200,000	7.49	8.4
9	2,290,000	6.36	9.0	5,600,000	6.75	9.0	17,900,000	7.25	8.9
9.5	1,740,000	6.24	9.4	6,900,000	6.84	9.3	437,000	5.64	9.3
10	740	2.87	9.5	420	2.62	9.4	35,000	4.54	9.6

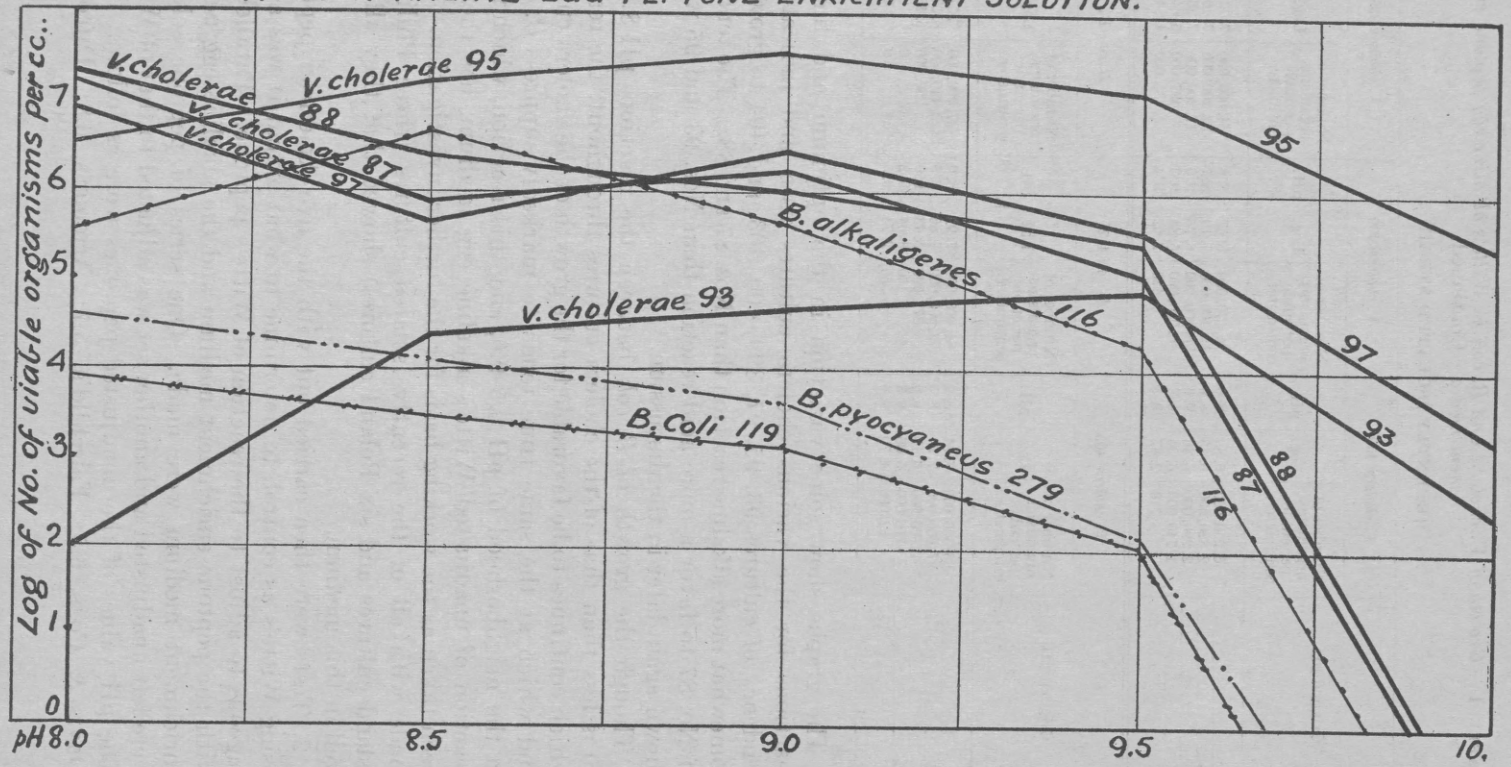
Original pH.	*V. choleræ* 405.			*B. coli* 412.			*B. coli* B.		
	Number of organisms per cubic centimeter.	Log.	pH.	Number of organisms per cubic centimeter.	Log.	pH.	Number of organisms per cubic centimeter.	Log.	pH.
8	86,300,000	7.94	8.2	84,000,000	7.92	8.0	37,000,000	7.57	8.8
8.5	87,000,000	7.94	8.5	72,000,000	7.86	8.5	3,410,000	6.53	8.9
9	1,560,000	6.19	9.0	271,000	5.43	9.0	280,000	5.45	9.2
9.5	116,000	5.06	9.3	1,100	3.04	9.2	0	0	9.3
10	62,000	4.79	9.6	550	2.74	9.5	0	0	9.4

The graphs show some variation in the optimum and limiting reactions for the various cholera cultures used and indicate the tendency of cultures 93, 95, 97, 399, 400, 403, and 404 to grow at a somewhat more alkaline reaction than Nos. 87 and 88. The tendency of No. 87 to favor a more acid reaction than Nos. 93 and 95 will be shown again later in the discussion.

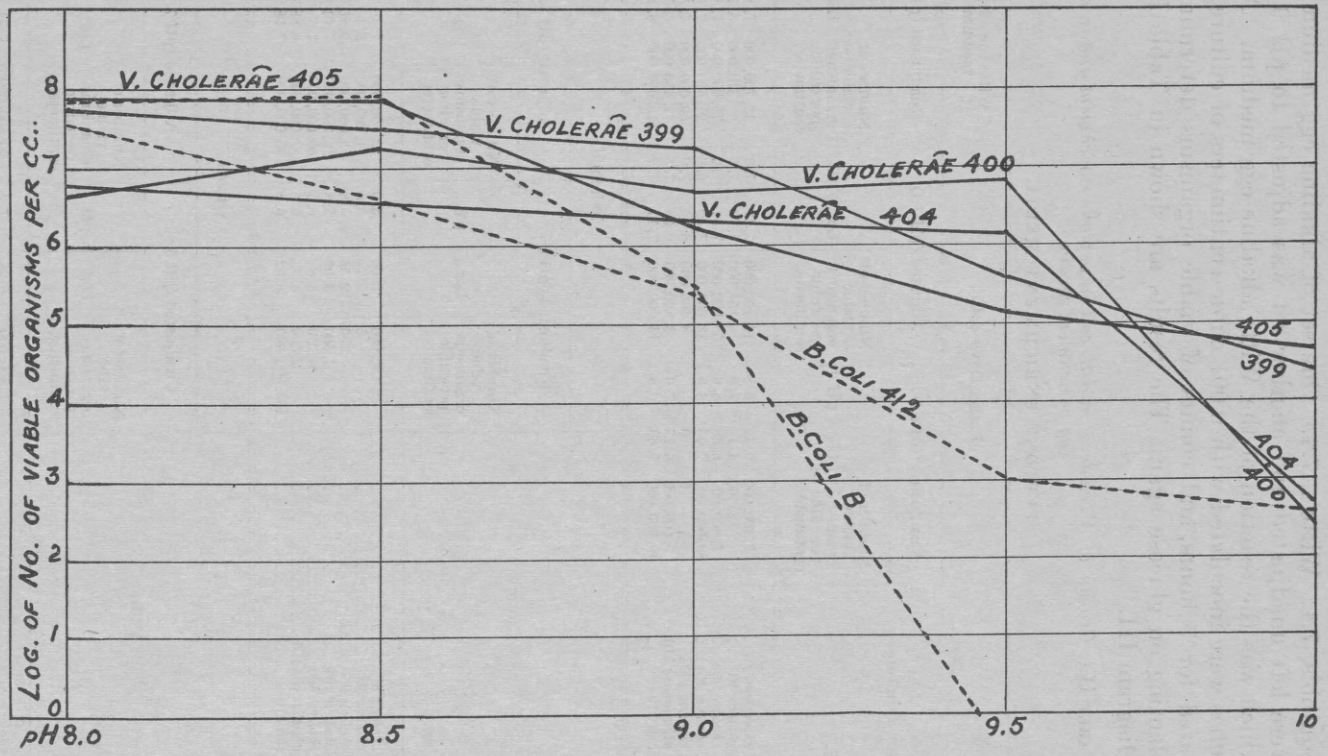
Though the growth of *B. coli* between the reactions pH 8.5 and 10 is less than that of the cholera cultures throughout, the reaction which continues to be favorable for the growth of the cholera cultures and which at the same time tends to markedly suppress *B. coli* is in the neighborhood of pH 9.5–9.6, and this reaction, which is the reaction of unadjusted Witte's alkaline egg medium, has therefore been taken as the working basis for the further investigation. Tests made with all of the recently isolated cultures (three Philippine Island cultures and six Poland cultures) showed that they all grew well in this medium.

2. Tests were then carried out with the five American peptones, using Witte's as control, to determine to what extent it was advantageous to adjust to the reaction of Witte's peptone. Parallel tests with the peptone enrichment medium and the alkaline egg peptone enrichment medium were made. One series of peptone solutions were left unadjusted and another set was adjusted to that of Witte's. The pH values of the unadjusted peptones were as follows: Parke, Davis & Co.'s, 6.5; Fairchild's, 5.7; Armour's, 6.8; Difco, 7.0;

DIAGR.I:- GROWTH OF V. CHOLERAE, B. COLI, B. ALKALIGENES AND B. PYOCYANEUS IN WITTE'S ALKALINE EGG PEPTONE ENRICHMENT SOLUTION.



DIAGR. II :- GROWTH OF V. CHOLERÆ AND B. COLI (RECENTLY ISOLATED CULTURES) IN WITTE'S ALKALINE EGG PEPTONE ENRICHMENT SOLUTION.



Squibb's, 7.4; Witte's, 7.4. One set of alkaline egg medium tubes were left unadjusted and another set was adjusted to pH 9.5–9.6, which was the reaction of the Witte alkaline egg medium. All the tubes were inoculated with 0.001 cubic centimeters of culture, incubated for 7 hours, and counts of viable organisms determined by planting on glucose agar. The results are shown in Table II and Diagram III.

TABLE II.—*Growth of V. choleræ and B. coli in peptone enrichment and in alkaline egg enrichment media.*

PEPTONE ENRICHMENT MEDIA.

Culture.	Parke, Davis & Co.						Witte (no adjusted medium).		
	Unadjusted (pH 6.5).			Adjusted (pH 7.4).			Unadjusted (pH 7.4).		
	Number of viable organisms per cubic centimeter.	Log.	pH.[a]	Number of viable organisms per cubic centimeter.	Log.	pH.	Number of viable organisms per cubic centimeter.	Log.	pH.
V. choleræ 87	3, 100, 000	6. 49	6. 8	14, 700, 000	7. 17	7. 3	12, 700, 000	7. 1	7. 4
V. choleræ 93	12, 500	4. 1	7. 0	54, 000	4. 73	7. 5	310, 000	5. 49	7. 1
V. choleræ 95	7, 600, 000	6. 88	6. 6	65, 000, 000	7. 81	7. 0	75, 000, 000	7. 88	7. 2
V. choleræ 404	89, 000, 000	7. 95	6. 0	33, 000, 000	7. 52	6. 8	25, 600, 000	7. 41	7. 3
V. choleræ 400	0	0	------	8, 400, 000	6. 92	7. 0	50, 000, 000	7. 7	7. 6
B. alkaligenes 116	5, 600, 000	6. 75	7. 0	3, 800, 000	6. 58	7. 5	2, 450, 000	6. 39	7. 2
B. coli 119	49, 000, 000	7. 69	6. 8	13, 000, 000	7. 11	7. 2	115, 000, 000	8. 1	7. 2

Culture.	Fairchild.					
	Unadjusted (pH 5.7).			Adjusted (pH 7.4).		
	Number of viable organisms per cubic centimeter.	Log.	pH.	Number of viable organisms per cubic centimeter.	Log.	pH
V. choleræ 87	1, 150, 000	6. 06	6. 0	12, 500, 000	7. 1	7. 6
V. choleræ 93	5	0. 7	5. 8	1, 260, 000	6. 1	7. 2
V. choleræ 95	350	2. 54	5. 8	73, 000, 000	7. 86	7. 5
V. choleræ 404	4, 600	3. 66	5. 8	10, 300, 000	7. 01	7. 1
V. choleræ 400	6, 250	3. 8	6. 2	50, 000, 000	7. 7	7. 2
B. alkaligenes 116	2, 500	3. 4	5. 8	7, 600, 000	6. 88	7. 5
B. coli 119	105, 000, 000	8. 02	6. 0	95, 000, 000	7. 98	7. 3

Culture.	Armour.					
	Unadjusted (pH 6.8).			Adjusted (pH 7.4).		
	Number of viable organisms per cubic centimeter.	Log.	pH.	Number of viable organisms per cubic centimeter.	Log.	pH.
V. choleræ 87	1, 890, 000	6. 28	7. 2	20, 500, 000	7. 31	7. 5
V. choleræ 93	40, 000	4. 6	6. 8	5, 450, 000	6. 74	7. 2
V. choleræ 95	5, 150, 000	6. 71	6. 8	27, 200, 000	7. 43	7. 3
V. choleræ 404	10, 500, 000	7. 02	6. 8	35, 000, 000	7. 54	7. 4
V. choleræ 400	5, 150, 000	6. 71	6. 8	15, 900, 000	7. 2	7. 2
B. alkaligenes 116	450, 000	5. 65	6. 8	1, 020, 000	6. 01	7. 4
B. coli 119	5, 070, 000	6. 71	6. 8	25, 900, 000	7. 41	7. 4

[a] After incubation.

TABLE II.—*Growth of V. choleræ and B. coli in peptone enrichment and in alkaline egg enrichment media*—Continued.

PEPTONE ENRICHMENT MEDIA—Continued.

Culture.	Difco.						Squibbs (no adjusted media).		
	Unadjusted (pH 7.0).			Adjusted (pH 7.4).			Unadjusted (pH 7.4).		
	Number of viable organisms per cubic centimeter.	Log.	pH.	Number of viable organisms per cubic centimeter.	Log.	pH.	Number of viable organisms per cubic centimeter.	Log.	pH.
V. choleræ 87	725,000	5.86	7.6	38,000	5.58	7.8	15,800,000	7.2	7.6
V. choleræ 93	39,000	4.59	7.0	2,200	3.34	7.3	22,500	4.35	7.2
V. choleræ 95	3,100,000	6.49	7.5	16,200,000	6.67	7.8	660,000	5.82	7.4
V. choleræ 404	14,100,000	7.15	7.4	------	------	------	87,000,000	7.94	7.0
V. choleræ 400	38,000,000	7.58	7.4	------	------	------	16,100,000	7.21	7.0
B. alkaligenes 116	21,000	4.32	7.0	380,000	5.58	7.5	53,000	4.72	7.5
B. coli 119	8,400,000	6.92	7.5	11,300,000	7.05	7.6	23,100,000	7.36	7.2

ALKALINE EGG ENRICHMENT MEDIA.

Culture.	Parke, Davis & Co.						Witte (no adjusted media).		
	Unadjusted (pH 9.4).			Adjusted (pH 9.6).			Unadjusted (pH 9.6).		
	Number of viable organisms per cubic centimeter.	Log.	pH.	Number of viable organisms per cubic centimeter	Log.	pH.	Number of viable organisms per cubic centimeter.	Log.	pH.
V. choleræ 87	30,000	4.48	9.0	⟨10,000	⟨4	9.2	8,250,000	6.92	9.2
V. choleræ 93	90,000	4.95	9.2	170,000	5.23	9.4	33,900	4.53	9.4
V. choleræ 95	2,800,000	6.45	9.0	9,300,000	6.97	9.2	65,000,000	7.81	9.3
V. choleræ 404	26,000,000	7.42	8.8	14,500,000	7.16	8.9	14,600,000	7.16	9.2
V. choleræ 400	11,300,000	7.05	8.9	7,100,000	6.85	8.9	11,700,000	7.07	9.2
B. alkaligenes 116	39,000	4.59	9.3	1,050	3.02	9.4	81,000	4.99	9.6
B. coli 119	⟨100	⟨2	9.3	⟨100	⟨2	9.4	⟨100	⟨2	9.6

Culture.	Fairchild.					
	Unadjusted (pH 8.9).			Adjusted (pH 9.6).		
	Number of viable organisms per cubic centimeter.	Log.	pH.	Number of viable organisms per cubic centimeter.	Log.	pH.
V. choleræ 87	19,600,000	7.29	8.4	28,000,000	7.45	8.7
V. choleræ 93	1,400	3.15	8.6	2,600	3.45	9.0
V choleræ 95	20,800,000	7.32	8.2	54,000,000	7.73	8.7
V. choleræ 404	10,800,000	7.03	8.8	3,900,000	6.59	9.2
V. choleræ 400	51,000,000	7.71	8.8	2,230,000	6.35	9.2
B. alkaligenes 116	183,000	5.26	8.6	20,500	4.31	8.8
B. coli 119	73,000	4.86	8.4	850	2.93	9.0

TABLE II.—*Growth of V. choleræ and B. coli in peptone enrichment and in alkaline egg enrichment media*—Continued.

ALKALINE EGG ENRICHMENT MEDIA—Continued.

Culture.	Armour.					
	Unadjusted (pH 9.2).			Adjusted (pH 9.6).		
	Number of viable organisms per cubic centimeter.	Log.	pH.	Number of viable organisms per cubic centimeter.	Log.	pH.
V. choleræ 87	15,500,000	7.19	8.8	35,000,000	7.54	9.2
V. choleræ 93	77,000	4.89	9.0	500	2.7	9.3
V. choleræ 95	4,550,000	6.66	8.8	33,000,000	7.52	9.2
V. choleræ 404	17,200,000	7.24	8.9	25,000,000	7.4	9.2
V. choleræ 400	7,300,000	6.86	8.9	4,450,000	6.65	9.2
B. alkaligenes 116	470,000	5.67	9.0	67,000	4.83	9.3
B. coli 119	2,900	3.46	9.0	⟨100	⟨2	9.3

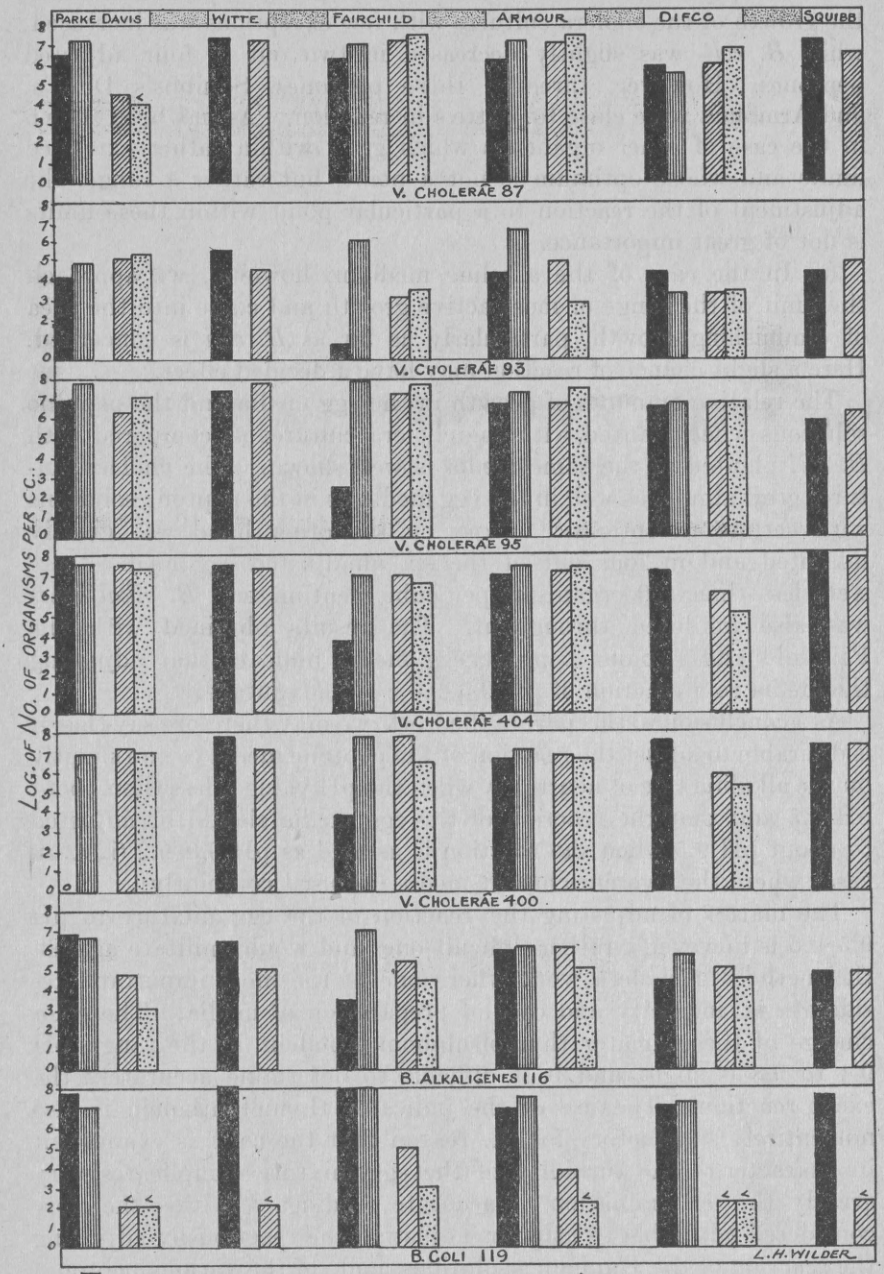
Culture.	Difco.						Squibbs (no adjusted media).		
	Unadjusted (pH 9.3).			Adjusted (pH 9.6).			Unadjusted (pH 9.6).		
	Number of viable organisms per cubic centimeter.	Log.	pH.	Number of viable organisms per cubic centimeter.	Log.	pH.	Number of viable organisms per cubic centimeter.	Log.	pH
V. choleræ 87	1,080,000	6.03	8.6	4,450,000	6.65	9.2	3,730,000	6.57	9.2
V. choleræ 93	1,200	3.3	9.2	2,900	3.46	9.4	56,000	4.75	9.3
V. choleræ 95	5,200,000	6.72	8.7	5,100,000	6.71	9.0	1,850,000	6.27	9.2
V. choleræ 404	1,410,000	6.15	9.3	106,000	5.1	9.5	52,600,000	7.72	9.2
V. choleræ 400	545,000	5.74	9.3	108,000	5.1	9.5	13,500,000	7.13	9.2
B. alkaligenes 116	117,000	5.07	9.2	19,600	4.29	9.4	45,500	4.66	9.4
B. coli 119	⟨100	⟨2	9.2	⟨100	⟨2	9.3	⟨100	⟨2	9.5

NOTE 1.—Parke, Davis & Co.'s peptone used for *V. choleræ* 400 and 404 had a reaction of pH 5.6.
NOTE 2.—The reaction of the alkaline egg medium in the case of cultures 400 and 404 was adjusted by adjusting the reaction of the Dunham's solution before the addition of the egg mixture.

The deductions to be drawn from the table and diagram are:

(*a*) That all the cholera cultures grew well in unadjusted peptone enrichment media made with the various peptones with the exception of Fairchild's and Parke, Davis & Co.'s (in one case). There was very scant growth in Fairchild's in the case of culture 93 (5 per cubic centimeter) and also in the case of culture 95 (350 per cubic centimeter). Cultures 400 and 404 also grew less readily in the unadjusted than in the adjusted medium. Culture 87, however, grew well in this medium at a reaction of pH 5.7. By the adjustment of the reaction of this peptone to pH 7.4, culture 93 showed 1,000,000 organisms per cubic centimeter and culture 95 showed 73,000,000 per cubic centimeter. There was no growth of culture 400 in an unadjusted Parke, Davis & Co.'s peptone (pH 5.6). In the same medium adjusted to pH 7.4 there were 8,400,000 organisms per cubic centimeter.

DIAGR. III:- GROWTH OF V. CHOLERAE, B. COLI AND B. ALKALIGENES IN PEPTONE AND ALKALINE EGG ENRICHMENT SOLUTIONS.



PEPTONE ENRICHMENT SOLUTION (REACTION UNADJUSTED).
" " " (" ADJUSTED TO pH 7.2).
ALKALINE EGG ENRICHMENT SOLUTION (REACTION UNADJUSTED).
" " " " (" ADJUSTED TO pH 9.6).

(*b*) There was a certain advantage in adjusting the reaction of all the other peptones to that of Witte's, viz, pH 7.2–7.4, inasmuch as the growth of the cholera cultures with few exceptions was increased, while *B. coli* was slightly decreased in two out of four adjusted peptones. However, three of these peptones, Squibbs's Difco's, and Armour's were close to Witte's in reaction. As has been shown in the case of other organisms which grow within rather extended limits and whose optimum is not a point, but rather a range, the adjustment of the reaction to a particular point within these limits is not of great importance.

(*c*) In the case of the alkaline medium, however, we approach the limit of the range of most active growth and come into the area of diminishing growth, particularly as far as *B. coli* is concerned. Here a slight chance of reaction may have a decided effect.

The relative amounts of growth in the egg media and the peptone solutions when planted with the cholera cultures as compared with *B. coli* planted in the same media is well shown. The cholera cultures grew almost as well in the egg media as in the peptone solutions with certain exceptions. *B. coli* on the other hand was greatly inhibited and in four out of the six unadjusted egg media there were less than 100 organisms per cubic centimeter. *B. alkaligenes* was also inhibited throughout. The results obtained with the Fairchild and Armour peptone egg media indicate that adjusting the media to a reaction of pH 9.5–9.6 is an advantage.

As a conclusion to this part of the work, we may therefore say that it is desirable to adjust the reaction of the peptone media to one slightly on the alkaline side of neutrality when the pH value is less than about pH 6.5 and that the reaction of the egg media should be adjusted to about pH 9.5 when the reaction is as acid as pH 9.0–9.2 (i. e., in cases where the two enrichment media are used conjointly).

The matter of adjusting the reaction of the egg mixture to pH 9.5–9.6 is however a rather difficult one, and would militate against the method in cholera work where one of the most important desiderata is simplicity and ease of preparation of media. The color change of the indicator thymolsulphomphthalein in the range pH 9.4 to 9.8 is slight and it is difficult to determine accurately the exact reaction. The use of the indicator thymolphthalein is also not entirely satisfactory for the reason that the color is evanescent in character. The turbidity of the egg mixture complicates very greatly the determination of accurate readings. It was therefore considered advisable to attempt adjusting the reaction by adjusting the reaction of the Dunham's solutions made of the various peptones to the reaction of Witte's, viz, pH 7.2, before the addition of the egg mixture. Owing to the different buffer properties of the various

peptones it is not to be expected that exactly the same alkaline reaction would be attained by this method. The Witte's and Squibbs's mixtures were always more alkaline than the others. In order to determine in actual practice how much difference there would be in the use of media adjusted according to the two different methods, tests were carried out with Parke, Davis & Co., Fairchild, Armour, and Difco media adjusted before and after the addition of the egg mixture. The results are shown in Table III and Diagram IV. As before the tubes of media were planted with 0.001 cubic centimeter of culture and incubated for 7 hours after which the colony count was determined by plating on glucose agar.

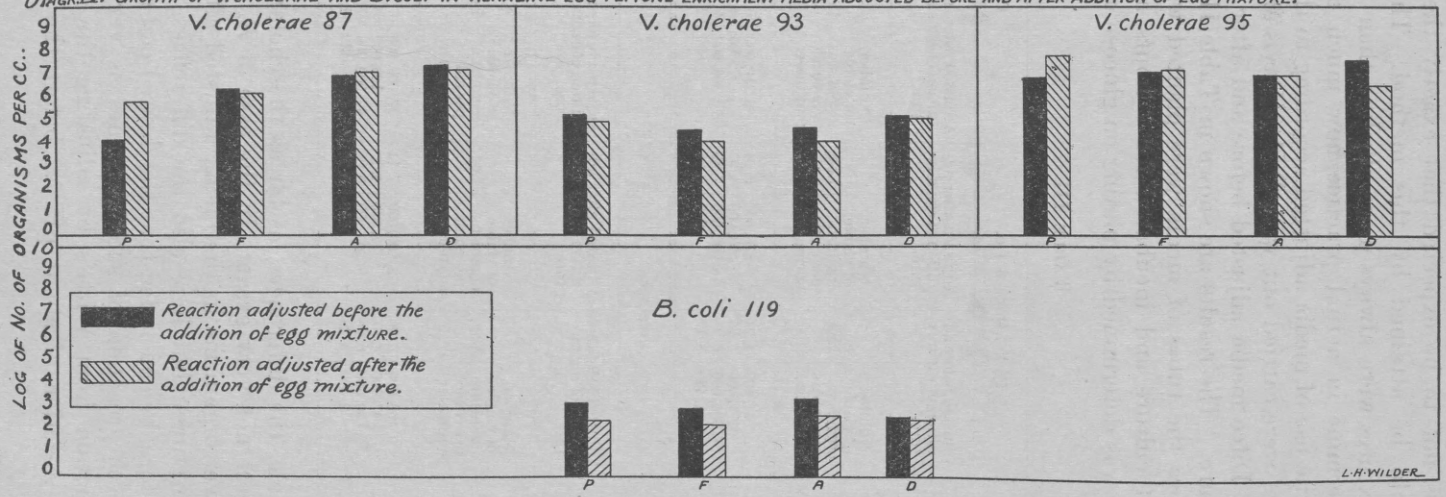
TABLE III.

Culture.	Parke, Davis & Co.				Fairchild.			
	Adjusted before addition of egg mixture.		Adjusted after addition of egg mixture.		Adjusted before addition of egg mixture.		Adjusted after addition of egg mixture.	
	Number of viable organisms per cubic centimeter.	Log.	Number of viable organisms per cubic centimeter.	Log.	Number of viable organisms per cubic centimeter.	Log.	Number of viable organisms per cubic centimeter.	Log.
V. choleræ 87	17,400	4.24	870,000	5.94	2,310,000	6.36	1,690,000	6.23
V. choleræ 93	177,000	5.25	93,000	4.97	37,500	4.57	11,600	4.06
V. choleræ 95	7,900,000	6.9	78,000,000	7.89	12,300,000	7.09	14,300,000	7.16
B. coli 119	1,480	3.17	230	2.36	890	2.95	210	2.32

Culture.	Armour.				Difco.			
	Adjusted before addition of egg mixture.		Adjusted after addition of egg mixture.		Adjusted before addition of egg mixture.		Adjusted after addition of egg mixture.	
	Number of viable organisms per cubic centimeter.	Log.	Number of viable organisms per cubic centimeter.	Log.	Number of viable organisms per cubic centimeter.	Log.	Number of viable organisms per cubic centimeter.	Log.
V. choleræ 87	9,100,000	6.96	12,800,000	7.11	27,600,000	7.44	16,200,000	7.21
V. choleræ 93	47,000	4.67	12,500	4.1	163,000	5.21	125,000	5.1
V. choleræ 95	9,600,000	6.98	9,200,000	6.96	47,000,000	7.67	4,300,000	6.63
B. coli 119	2,400	3.38	530	2.72	430	2.62	310	2.49

On the whole, the differences, as far as the cholera cultures were concerned, were not very great. In the case of *B. coli*, there were uniformly fewer organisms in the media adjusted after the addition of the egg mixtures; but here also the differences were not great. It may, therefore, be concluded that the desired end may be attained by adjusting the reaction of such peptones as vary to any great extent in reaction from Witte's by adjusting the base Dunham's solution to a reaction of about pH 7.2 before the addition of the egg mixture.

DIAGR. IV:- GROWTH OF V. CHOLERAE AND B. COLI IN ALKALINE EGG PEPTONE ENRICHMENT MEDIA ADJUSTED BEFORE AND AFTER ADDITION OF EGG MIXTURE.



3. Further information on the growth of *V. choleræ* and *B. coli* in the enrichment media was sought by planting with mixtures of the two organisms. Tubes of peptone solution and adjusted and unadjusted alkaline egg enrichment media were planted with the following combinations:

V. choleræ 87 (0.001 cubic centimeter)–*B. coli* 119 (0.1 cubic centimeter).

V. choleræ 93 (0.005 cubic centimeter)–*B. coli* 119 (0.1 cubic centimeter).

V. choleræ 95 (0.001 cubic centimeter)–*B. coli* 119 (0.1 cubic centimeter).

After 7 hours incubation the growth was streaked on Endo plates to determine the relative proportion of the two kinds of colonies. The results with culture 95 are shown in Table IV.

TABLE IV.—*Peptone enrichment and alkaline egg peptone enrichment solution planted with V. choleræ 95 (0.001 cc) and B. coli 119 (0.1 cc) streaked on Endo plates.*

	Alkaline egg enrichment solution.				Peptone enrichment solution.	
	Reaction unadjusted.		Reaction adjusted to pH 9.5.		Reaction unadjusted.	
	Number of *V. choleræ*.	Number of *B. coli*.	Number of *V. choleræ*.	Number of *B. coli*.	Number of *V. choleræ*.	Number of *B. coli*.
Parke, Davis & Co.'s peptone:						
Plate 1	Numerous	550	Numerous	75	Colonies indistinguishable.	Numerous.
Plate 2	350	38	650	0	75	500.
Plate 3	48	3	42	1	0	191.
Witte's peptone:						
Plate 1	550	12	No adjusted medium.		Colonies indistinguishable.	Numerous.
Plate 2	1	0			3	750.
Plate 3	0	0			1	42.
Fairchild's peptone[a]						
Plate 1	Numerous	Numerous	Numerous	250	Numerous	Numerous.
Plate 2	330	250	135	14	390	620.
Plate 3	35	26	19	7	65	125.
Armour's peptone:						
Plate 1	1,200	238	Numerous	75	Colonies indistinguishable.	Numerous.
Plate 2	0	0	78	1	16	120.
Plate 3	0	0	0	0	12	112.
Difco peptone:						
Plate 1	1,200	210	1,500	450	Numerous	Numerous.
Plate 2	35	115	350	42	127	550.
Plate 3	5	9	8	2	0	33.
Squibb's peptone:						
Plate 1	Numerous	25	No adjusted medium.		Colonies indistinguishable.	Numerous.
Plate 2	208	3			3	750.
Plate 3	9	0			1	42.

[a] Reaction of peptone enrichment medium adjusted to pH 7.4.

The advantage of using the alkaline egg medium for enrichment of *V. choleræ* is indicated by the greater number of colonies of *V. choleræ* and the small number of *B. coli* colonies occurring on the plates streaked with these cultures as contrasted with the small number of *V. choleræ* and large number of *B. coli* streaked with the peptone solution cultures. The results obtained with the adjusted alkaline egg peptone solution also are more favorable for the isolation of *V. choleræ* than the unadjusted egg medium.

The results with cultures 87 and 93 were similar. Colonies of 93 were indistinguishable on all of the plates streaked with the peptone solution cultures except in the case of Parke Davis peptone.

4. Alkaline egg agar medium. Tests were carried out to determine what was the reaction of Witte's alkaline egg agar favorable for the growth of *V. choleræ* and inhibitory to *B. coli*. The medium was made according to the Goldberger formulæ. One portion of agar base was left unadjusted, another was adjusted to pH 7.0, and a third portion was adjusted to pH 7.4 before the addition of the egg mixture. The plates were streaked with cholera cultures 87, 93, 95, and 97 and *B. coli* 119. The results are shown in Table V.

TABLE V.—*Growth of V. choleræ and B. coli on Witte's alkaline egg agar.*

Reaction of agar base.	*V. choleræ* 87		*V. choleræ 93.*		*V. choleræ 95.*		*V. choleræ* 97.		*B. coli 119.*	
	Number of colonies.	Size.	Number of colonies.	Size.	Number of colonies.	Size.	Number of colonies.	Size.	Number of colonies.	Size.
Unadjusted pH, 5.8	650	[1] 90	580	55	880	75	650	110	[2] 850	22
Adjusted pH, 7.0	850	85	254	55	740	74	680	110	[3] 0	0
Adjusted pH, 7.4	920	90	655	55	870	65	830	90	[4] 0	0

[1] Divisions of micrometer scale (50=1 millimeter).
[2] Distinct colonies.
[3] Several areas of hazy growth. Very minute pin-point colonies.
[4] No growth.

By adjusting the reaction of the agar base to pH 7 before the addition of the egg mixture a medium was obtained which almost completely prevented the development of *B. coli*. By adjustment to pH 7.4 no growth whatever was visible. The cholera colonies were, however, as numerous and as large as on the unadjusted plates.

5. An adjustment of the agar base to a reaction in the neighborhood of pH 7.4 was, therefore, in accordance with the above results, considered a favorable one and tests were then carried out with the various other peptones, and comparisons obtained of growth on unadjusted agar and agar adjusted to the above reactions. The results are presented in Table VI.

TABLE VI.—*Growth of V. choleræ, B. coli and B. alkaligenes on unadjusted and adjusted alkaline egg agar.*

	pH of agar base.	*V. choleræ* 87.		*V. choleræ* 90.		*V. choleræ* 93.	
		Number of colonies.	Size.[a]	Number of colonies.	Size.	Number of colonies.	Size.
Parke, Davis & Co.'s:							
Unadjusted	5.8	680	140	410	150	495	90
Adjusted	7.4	550	150	232	160	450	65
Witte's:							
Unadjusted	6.4	116	100	610	125	178	17–60
Adjusted	7.4	352	125	350	125	112	11–60
Fairchild's:							
Unadjusted	5.4	750	130	400	150	44	15–90
Adjusted	7.4	1,200	130	750	110	0	------
Armour's:							
Unadjusted	6.4	79	150	334	140	198	20–70
Adjusted	7.4	145	170	97	160	129	20–50
Difco:							
Unadjusted	6.4	500	100	72	160	129	15–80
Adjusted	7.4	540	130	39	160	62	10–70
Squibb's:							
Unadjusted	6.6	680	125	236	160	[b] 0	15–65
Adjusted	7.4	219	140	204	160	[c] 0	30

	pH of agar base.	*V. choleræ* 95.		*V. choleræ* 400.		*B. alkaligenes* 116.		*B. coli* 119.	
		Number of colonies.	Size.	Number of colonies.	Size.	Number of colonies.	Size.	Number of colonies.	Size.
Parke, Davis & Co.'s:									
Unadjusted	5.8	1,100	105	630	120	[d]	[e]	1,250	30
Adjusted	7.4	410	115	850	90	0	------	0	------
Witte's:									
Unadjusted	6.4	720	110	144	110	0	------	126	30
Adjusted	7.4	750	90	410	110	0	------	0	------
Fairchild's:									
Unadjusted	5.4	760	125	30	130	24	22	1,500	40
Adjusted	7.4	630	90	45	130	0	------	0	------
Armour's:									
Unadjusted	6.4	435	110	125	150	[f] 0	8	125	18
Adjusted	7.4	224	75	480	120	0	------	0	------
Difco:									
Unadjusted	6.4	390	100	720	125	0	------	8	11
Adjusted	7.4	56	110	1,200	125	0	------	0	------
Squibb's:									
Unadjusted	6.6	890	110	560	150	0	------	80	22
Adjusted	7.4	730	105	830	125	0	------	0	------

[a] Divisions of micrometer 50=1 millimeter.
[b] 117 on first plate.
[c] 13 on first plate.
[d] Numerous.
[e] Pin point.
[f] 89 on first plate.

The advantage of adjusting the agar to a reaction of pH 7.4 is shown by comparing the results obtained with *B. coli* on adjusted and unadjusted media. In all cases there was a growth on the unadjusted plates, and none on the adjusted plates. The size of the colonies of *B. coli* on the unadjusted media was small in comparison with the size of the cholera colonies and would perhaps not interfere with the isolation of *V. choleræ*, but the advantage of adjusting the medium to a more alkaline reaction is nevertheless evident.

6. Isolation of *V. choleræ* from artificially contaminated stools.

Media for the isolation of the organism were prepared in accordance with the information obtained in the above tests. Peptone

enrichment media were unadjusted with the exception of Fairchild's and Parke, Davis & Co.'s, which were adjusted to pH 7.4. The alkaline egg enrichment media were prepared by adjusting the base Dunham's solution to a reaction of pH 7–7.2 and the agar adjusted to about pH 7.4 before the addition of the egg mixture.

A sample of stool was emulsified, strained through sterile gauze and divided into several parts, one of which was left uncontaminated to serve as control and two other portions were contaminated with cholera cultures as follows:

4 cubic centimeters feces emulsion—1 cubic centimeter broth culture *V. choleræ* 87.

4 cubic centimeters feces emulsion—1 cubic centimeter broth culture *V. choleræ* 95.

4 cubic centimeters feces emulsion—1 cubic centimeter broth culture *V. choleræ* 400.

0.1 cubic centimeter of the mixtures were planted in the two enrichment media, incubated for 7 hours and streaked on the alkaline egg agar plates. The results obtained with cultures 95 and 400 are indicated in Table VII. Similar results were obtained with culture No. 87. Efforts to isolate cholera culture No. 93 were not as successful. By using a comparatively large amount of the culture, it was, however, isolated from Parke, Davis & Co., Witte, and Difco media. No colonies which could be identified as *V. choleræ* were fished from plates made with Fairchild, Armour, or Squibb's peptones. This organism seems to be an aberrant one in some respects and throughout this work always failed to grow as readily as the other organisms employed.

TABLE VII.—*Alkaline egg agar—Isolation of V. choleræ from contaminated stools.*

	Peptone enrichment solution.				Alkaline egg enrichment solution.[a]			
	V. choleræ 95.		Other colonies.		*V. choleræ* 95.		Other colonies.	
	Number.	Size.	Number.	Size.	Number.	Size.	Number.	Size.
Parke, Davis & Co.	625	85	92	24	465	85	164	24
Witte	(b)	50	(c)	------	475	65	105	16
Fairchild	[d] 1,200	65	55	35	111	74	175	46
Armour	[d] 1,200	52	46	18	132	53	196	44
Difco	[d] 1,000	46	25	16	220	56	82	37
Squibb's	[d] 1,500	54	13	17	178	67	72	21
	V. choleræ 400.		Other colonies.		*V. choleræ* 400.		Other colonies.	
	Number.	Size.	Number.	Size.	Number.	Size.	Number.	Size.
Parke, Davis & Co.	265	100	62	60	136	110	61	70
Witte	460	65	0	------	525	90	95	45
Fairchild	18	90	325	60	53	100	124	75
Armour	335	110	82	70	44	90	150	50
Squibb's	485	110	135	55	315	120	53	50

[a] Witte's and Squibb's peptones required no adjustment in the enrichment media.
[b] Very numerous.
[c] Not distinguishable.
[d] Number estimated.

Considering Table VII, which represents an application of the results obtained in the previous work, it appears that the results with Witte's peptone were somewhat more favorable than those obtained with the other peptones in that on those plates streaked with the growth in peptone enrichment solution the *B. coli* colonies were indistinguishable or absent and the number of cholera colonies was very large. In no case, however, was there any difficulty in distinguishing cholera colonies on the other plates, as they were usually more numerous and larger than the other colonies. The cholera colonies are more transparent and darker in color than those of *B. coli*, which are white and opaque. In all the previous work no particular advantage had been observed in the use of Witte's peptone over the other peptones, except for the fact that this peptone, together with Squibb's, were sufficiently alkaline to require no adjustment of reaction in the alkaline egg enrichment fluid.

In the test with culture 95 the results with the peptone solution apparently are more favorable than with the alkaline egg enrichment medium. The amount of contamination of *V. choleræ* was comparatively large, and smaller amounts would probably show more the advantage of the alkaline egg enrichment fluid. Such a test, in which the contamination with the cholera organism was one-tenth the volume of feces emulsion instead of one-fifth, showed equally good results on plates made from the two enrichment fluids, all the plates showing almost pure cultures of *V. choleræ*, except in the case of Fairchild's where the cholera colonies and the other colonies were about equally divided.

SUMMARY.

In conclusion it may be stated that the American peptones in general are suitable for the isolation of *V. choleræ*. Good growth was obtained in all media with the exception of Fairchild's peptone solution and sometimes Parke, Davis & Co.'s peptone solution, which were found to be too acid. These by adjustment to a more alkaline reaction could be made favorable.

The results of this work do not show any great advantage of one particular peptone over the others.[5] The Parke, Davis & Co. peptone when properly adjusted, appeared to furnish more luxuriant growths than some of the others, perhaps on account of suitable amino acids. Squibb's peptone offers the advantage of a reaction as alkaline as Witte's, and less adjustment of reaction was necessary than in the

[5] The tests reported in this paper were carried out with one peptone of each manufacturer, except in the case of Parke, Davis & Co. in which three were used. The peptones used were received at the laboratory two or more years before the date of this paper. It was not possible to carry out tests with a number of peptones of each manufacturer. The results obtained should not therefore necessarily be interpreted as applying to all the peptones of one manufacturer, as it is recognized that different lots of peptones from the same manufacturer vary to some extent.

case of some of the other peptones. Good results were obtained with Difco peptone, which was only slightly more acid than Witte's. Armour's peptone throughout showed a tendency to favor *B. coli* somewhat more than those just enumerated. Fairchild's peptone appeared on the whole to be least suitable in that it was found to be more acid than the others and apparently some other elements were lacking even though the reaction was properly adjusted.

The general statement may be made in regard to reaction that owing to the variation among different peptones it is advisable to adjust to a more nearly uniform reaction, particularly in the case of those which vary to any considerable degree from Witte's in reaction.

If the two enrichment media are used conjointly it does not appear to be of any great advantage to adjust the reaction of the peptone solution to a definite point if the reaction is somewhere near neutrality. If the peptone solution alone is used for enrichment it would seem advisable to adjust to a rather alkaline reaction, though it can not be expected that a stable medium will be obtained without a buffer if the reaction is made more alkaline than about pH 8.

By adjusting the reaction of the Dunham's solution in the alkaline egg enrichment medium and of the agar in the alkaline egg plating medium to a point slightly on the alkaline side of neutrality, about pH 7.2–7.4, before the addition of the egg mixtures, suitable media are obtained. Adjustment of the reaction to this point is simple and definite, whereas adjustment to a uniform reaction after the addition of the egg mixture is too difficult and uncertain to be practical.

Most laboratories at the present time are equipped to adjust the reaction of culture media by the new method, and if the adjustment of the agar and Dunham's solution is made at the time of preparation there will be no unnecessary delay at the time the medium is to be used.

HYGIENIC LABORATORY BULLETINS OF THE PUBLIC HEALTH SERVICE.

The Hygienic Laboratory was established in New York, at the Marine Hospital on Staten Island, August, 1887. It was transferred to Washington, with quarters in the Butler Building, June 11, 1891, and a new laboratory building, located in Washington, was authorized by act of Congress March 3, 1901.

Of the bulletins published by the laboratory since its establishment copies of the following are available for distribution and may be obtained without cost by applying to the Surgeon General, United States Public Health Service, Washington, D. C.

No. 2.—Formalin disinfection of baggage without apparatus. By M. J. Rosenau.

No. 65.—Facts and problems of rabies. By A. M. Stimson.

No. 73.—The effect of a number of derivatives of choline and analogous compounds on the blood pressure. By Reid Hunt and R. de M. Taveau.

No. 78.—Report No. 4 on the origin and prevalence of typhoid fever in the District of Columbia (1909). By L. L. Lumsden and John F. Anderson. (Including articles contributed by Thomas B. McClintic and Wade H. Frost.)

No. 81.—Tissue proliferation in plasma medium. By John Sundwall.

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